Alginate Microspheres for Protein Delivery in Tissue Engineering

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ABSTRACT

Three-dimensional polymeric scaffolds have been widely used in tissue engineering for protein delivery. Scaffolds fabricated with different biomaterials and structures display various functions in protein delivery. A microsphere based delivery system is one sophisticated method. In this research, the potentials of alginate microspheres as protein carriers were tested. Alginate microspheres were prepared by a water-in-oil emulsion external gelation technique and loaded with bovine serum albumin (BSA) or DyLight 800 dye-labeled rabbit immunoglobulin G (IgG). Chitosan coated protein-loaded alginate microspheres were also prepared. The effects of process parameters on microsphere size, size distribution, encapsulation efficiency, and *in vitro* protein release profiles were investigated. Scanning electron microscopic photos showed that high dispersing force and high calcium chloride concentration produce small and uniform alginate microspheres with spherical shape and smooth surface. The release profiles indicated that BSA release from large and heterogeneous alginate microspheres was rapid and had a large initial burst release, and IgG release from small and homogeneous microspheres was slower and had lower initial burst release. Chitosan coating caused slower protein release compared to uncoated alginate microspheres in all cases. Protein-loaded microspheres were incorporated into alginate cylindrical scaffolds by long-term moulding in tubing for BSA or by fast gelation extruded from tubing for IgG. The scaffolds contained dried BSA loaded microspheres prepared using either protein incorporation or incubation methods, or with wet IgG-loaded microspheres with different concentrations. The release studies of BSA indicated that dried microspheres provided fast release possibly. The IgG release from scaffolds illustrated that chitosan coated IgG microspheres had more prolonged release profiles, and fast gelation of scaffolds could potentially eliminate protein loss during long-term gelation. All release profiles of scaffolds demonstrated that the initial burst effect was diminished and the release was extended by using a delivery system in which microspheres were incorporated into larger scaffolds. These prepared alginate microspheres and microsphere-incorporated scaffolds have been proven to have abilities of carrying and releasing proteins. Their applications toward delivery of functional proteins at the target site in patients for therapeutic purposes should be considered.

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LIST OF ABBREVIATIONS

Alg – alginate

bFGF – basic fibroblast growth factor

BSA – bovine serum albumin

CMC – critical micelle concentration

CS – chitosan

DMEM – Dulbecco's modified Eagle's medium

ECM – extracellular matrix

HA – hyaluronic acid

HCO-60 – hydrogenated castor oil 60

HLB – hydrophile-lipophile balance

IgG – immunoglobulin G

NGF – nerve growth factor

PBS – phosphate buffered saline

PCL – poly(caprolactones)

PEG – polyethylene glycol

PGA – poly(glycolic acid)

p*I* – isoelectric point

PLA – poly(lactic acid)

PLG – poly(lactide-co-glycolide)

PLGA – poly(lactic-co-glycolic acid)

PLLA – poly(L-lactic acid)

TBS – Tris-buffered saline

VEGF – vascular endothelial growth factor

Chapter 1. Introduction

1.1. Protein delivery in tissue engineering

Tissue engineering focuses on developing strategies to regenerate or repair damaged tissues or organs in patients. Numerous methods have been developed and tested *in vitro* and *in vivo* with the aim of either stimulating regrowth of damaged tissues or constructing new engineered man-made tissues. Regeneration of functional tissues and incorporation of new tissues require adhesion, migration, proliferation, and organization of new and old cells at the injured site. In order to maintain and regulate the complex cellular activities, delivery of signalling proteins, functional peptides, and therapeutic drugs may be needed. In recent years, progress in tissue engineering allows delivery of functional and active proteins to act as chemical cues for desired biological purposes.

Numerous proteins have been used in tissue engineering to serve a variety of functions. Proteins such as insulin, other hormones, and synthetic vaccines, have been widely used to manage poorly controlled diseases [1-3]. Growth factors are one type of protein that have abilities to regulate cell signalling, which can stimulate or inhibit growth of cells. Delivery of growth factors has become a valuable tool for directing cell proliferation, differentiation, migration, and angiogenesis in tissues [4-7]. The use of synthetic or conjugated peptides has also been proven to effectively guide and facilitate cellular activities owing to their improved penetration abilities into cells [8-10]. In order to exert the advantages of functional and therapeutic proteins in regenerative medicine, a suitable delivery system is required.

Delivery techniques used in tissue engineering generally involve incorporating desired proteins into polymeric matrices. Key parameters that should be considered when designing a protein-delivery vehicle include localization of delivered proteins, targeting appropriate cells, optimal time period for protein administration, and appropriate doses of protein needed at different times. The delivery mechanism should be carefully designed to achieve regulated protein release over preferred time intervals.

Protein release depends on the physicochemical and chemical properties of the polymer that are used to construct the carrier matrices as well as on the matrix structures. One of the fundamental release mechanisms is polymer-degradation-induced protein release. Hydrolytic or

enzymatic degradation of matrix polymers leads to cleavage of chemical bonds in matrix networks, which cause detachment of proteins from the internal networks. Another release mechanism is by diffusion of ions and molecules from polymeric matrices through the internal and external pores of the matrices. Many types of engineered delivery systems have been developed by utilizing different release mechanisms including the two mentioned above. A few types of these devices that are often used in tissue engineering are described as follows.

Early drug reservoir systems were designed by surrounding a compact drug core with a polymer membrane or coating. This type of system is usually made of biocompatible but non-degradable polymers [11]. Therefore, the drug release is dependent on the diffusion of the drug through the matrices. The rate of drug release is controlled by altering the size of the system and manipulating the drug concentration at the inner surface of the enclosed membrane [12]. The diffusion of drugs from these systems is normally slow, and can last for years. However, any unwanted rupture in the coating surfaces can cause a sudden increase in drug concentration, which potentially has toxic side effects for patients. Also, surgery can sometimes be required to remove the non-degradable system after drugs have been released.

Polymer matrices that incorporate uniformly distributed proteins are also commonly used for protein delivery. Both biodegradable and non-biodegradable polymers have been used to fabricate this type of delivery system. In the case of non-degradable polymer matrices, the release relies on the diffusion of proteins throughout the solid matrix networks. The diffusion rate of proteins is regulated by the thickness and porosity of the matrix, the initial loading concentration of proteins, and the solubility of the protein [13]. Molecules near the surface diffuse out first, while those trapped deeper inside the matrix must overcome the constrictions of interconnected cavities within the matrices to move near the surface [14]. It has been reported that the protein release rate from a non-degradable poly(ethylene-vinyl acetate) matrix increases as the total loading increases, and large proteins have difficulties diffusing through the solid matrices [14]. Similar to the reservoir systems, non-degradable matrices need to be surgically removed from the implanted site.

The biodegradable polymer matrices, on the other hand, are more applicable to human patients. Protein release from biodegradable matrices is not only dependent on the diffusion of protein, but also on the degradation rate of the polymers. Biodegradable polymers, such as

poly(lactide-co-glycolide) (PLG), poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and poly(lactic-co-glycolic acid) (PLGA), poly(caprolactones) (PCL), have become attractive polymers for protein delivery [15]. The degradation process of these materials involves hydrolysis of polymer backbones into non-toxic monomers. The protein release rate could be controlled by changing the degradation rate of polymers, which can be achieved by chemical modification of polymers [7].

The third common protein delivery method is accomplished through protein loaded hydrogels. A hydrogel can be fabricated into many geometrical configurations, for example, cylinders, slabs, disks, or spheres. The following section focuses on the use of hydrogel microspheres, which are referred to as macro-gels. Hydrogels made with synthetic and natural polymers usually have a porous surface and a complex internal network. The swelling property of hydrogels in water allows free movement of proteins throughout cavities in hydrogels. The rate of protein release from a hydrogel might be regulated by controlling the degree of hydration, the permeability, the pore size, the porosity, and increasing or decreasing the ionic or covalent interactions between proteins and hydrogels. Proteins could be loaded into hydrogels before or after scaffold preparation; each method has its own benefits and drawbacks. Incorporating proteins before scaffold construction requires hydrophilic biomaterials and non-toxic crosslinking formulations. Proteins can be evenly distributed in polymer solution and embedded in hydrogel matrices after crosslinking in this pre-loading method. However, only limited numbers of polymers and crosslinker can be used, since harsh preparation processes can destroy the stability and bioactivity of proteins. For instance, localized growth factor delivery was achieved by mixing growth factor protein with alginate solution then ionically gelling the mixture to form hydrogels. A previous study [16] showed that growth factors delivered *in vivo* have the ability to improve angiogenesis in tissues. On the other hand, incorporating proteins after formation of a hydrogel involves deposition and adsorption of proteins onto internal and external surfaces of hydrogels. This technique has the advantages of allowing optimization of hydrogel properties without compromising protein bioactivity. However, the quantity of protein loading that is achieved relies mainly on the chemical interaction and adsorptive properties of the protein and of the hydrogel surface. For example, protein which is ionically bonded to gelatin hydrogels can be controllably released according to the biodegradation rate of the gelatin hydrogels, which is in turn regulated by the extent of crosslinking of the hydrogels [17].

Although the rate and time span of protein delivery by hydrogels have a high dependence on the biomaterial composition and the construct of matrices, hydrogels are considered to have great potential as a protein delivery system because they are amenable to careful modifications and manipulations.

1.2. Three-dimensional tissue scaffolds

Tissue engineering has great potential in the development of strategies to restore tissue and cellular functions for tissues with limited regeneration ability. A revolutionary approach in tissue engineering to regenerate or repair tissue after injury is to use bioengineered three-dimensional polymer scaffolds. Such scaffolds have the ability to delivery growth factors, nutrients, and even living cells to the injured site, and also to provide mechanical strength to improve tissue development. The design of scaffolds suitable for tissue regeneration should meet the requirements or criteria of specific tissue types, as identified in the literature. The material property of the scaffolds is one critical factor. The scaffold materials should be biocompatible and not provoke any immune response in the tissue. The degradation profiles of the scaffold materials, which are linked to their physical and chemical properties, have to be easily adjusted or controlled to conform to the desired time span, but at the same time should provide the mechanical properties that are needed to support tissue growth. A desired material should also have the appropriate surface chemistry for cell attachment and proliferation. The micro- and macro- structures of scaffolds are also important factors dominating the functions of scaffolds. The macro-structure of scaffolds needs to be adjustable to a variety of configurations to fit the shape of interest. The porous surface and interconnected internal network of scaffolds should allow nutrient transport for cell survival, cell signalling, and ingrowth of vasculature and other tissues components. Scaffold properties should be tailored to the type of tissue to be regenerated. The microstructure or internal structure of scaffolds should be optimized to match the morphology and the growth properties of the tissues. For example, nerve regrowth requires longitudinal guidance, which is made possible by scaffolds with longitudinally orientated porous microstructure [18, 19].

Numerous attempts have been made to construct scaffolds that mimic the structure and biological functions of extracellular matrix (ECM), which is believed to have an optimal microenvironment for cell communication and cell signalling leading to regeneration of

functional tissues. Another function of ECM-mimicking scaffolds is to reduce the mechanical stress at the tissue-scaffold interface until the tissue is fully regenerated. Scaffolds utilizing both naturally derived polymers and biodegradable synthetic polymers have been widely used for tissue engineering applications. Many synthetic polymers have been used as scaffold materials, such as, PLA, PGA, PLG, PLGA, PCL, polypeptides, polyethylene glycol (PEG), and ceramics. Artificial polypeptides can be engineered to have desired amino acid sequences and structures, which allows modifications in scaffold stiffness and degradation rate [20]. Polyesters, including PLA, PGA, and PLGA, can be manipulated to produce different external and internal structures, and have been shown to have a good ability to promote cell attachment and long-term survival [21-24]. Porous scaffolds consisting of inorganic materials like ceramics and bioglass have great potential in bone tissue engineering due to their mechanical properties [25-27]. Naturally derived polymers have some biochemical properties that are very similar to the macromolecules produced by mammals, and therefore have the advantage of being recognized by the biological environment. However, technological manipulation of natural polymers is more complicated than it is for most synthetic polymers due to their complex structures.

The degradation mechanisms of naturally derived polymers are hydrolysis and/or biodegradation by enzymes produced by the host. These degradation mechanisms have two aspects. The advantage is that the degradation products can be excreted or resorbed by the metabolic processes of the host. This means that undegraded natural polymers can continue to offer specific biological functions over a period of time. The disadvantage is that the rate of degradation needs to be carefully controlled to allow the polymers to fulfill their biological functions within scaffolds within an optimal time frame. Techniques for controlling the degradation rate of natural polymer scaffolds have been studied, such as chemical crosslinking or structural modification of the scaffold matrix material.

The protein-based polymers, collagen, elastin, and fibrin, are considered to be superior biomaterials for scaffold design, since these proteins are often a main component of the ECM in many tissues. The ability of collagen and its derivatives to form sponge-like and gelatinous structures provide ECM-mimicking matrices for cell attachment and ingrowth, and their low physical strength has been improved by chemical crosslinking [28-30]. Because of its natural adhesive properties, the blood clot protein fibrin has gained a great deal of attention for use in

fabrication of tissue repair scaffolds. Fibrin forms gels through polymerization of fibrinogen in the presence of the protease enzyme thrombin, and gel degradation rate can be controlled by altering the concentration of a protease inhibitor. It has been reported that fibrin gels can promote cell migration, proliferation, and differentiation [31-33].

The polysaccharide-based polymers, such as hyaluronic acid (HA), chitosan, and alginate, are popular biomaterials, but their cell adhesive properties are usually lower than those of the protein-based polymers. HA is an acidic glycosaminoglycan commonly present in ECM, which can regulate cell motility and mediate cell differentiation. Various chemically modified HA derivatives have been developed to improve mechanical strength and cell attachment [34-36]. Chitosan has a structure similar to natural glycosaminoglycans, and can be degraded by chitosanase and lysozyme [37]. Chitosan is capable of forming porous scaffolds by ionic or chemical crosslinking, and its ability to promote cell attachment has been enhanced by combining it with proteins [38-40] or by covalently modifying the sugar residues along the chitosan backbone [41]. Alginate from brown algae has similar biochemical properties to chitosan, and could also be used as an efficient biomaterial for scaffold construction. However, alginate has lower biocompatibility with a mammalian host, which reduces its ability to promote cell differentiation, proliferation, and attachment [42, 43]. Nonetheless, the easy and fast gelation feature of alginate still attracts much attention for tissue engineering applications [44-46].

A wide range of synthetic and natural polymers has been explored for their abilities to construct three-dimensional scaffolds in tissue engineering. To fabricate a functional scaffold, the mechanical, chemical, and physicochemical features of the scaffolds that are produced have to be suitable for the type of tissue to be regenerated and for the biological environment into which they will be implanted. The practical advantages of a porous three-dimensional scaffold are undeniable, and with careful design, they have great potential to guide cells toward successful tissue regeneration.

1.3. Microspheres as protein carriers

One way of delivering bioactive molecules and achieving sustained release is the use of microsphere-based delivery systems [47-49]. Microspheres used as a protein delivery system should encapsulate the desired amount of protein, protect the integrity of protein functions,

induce no immune responses, and transport intact proteins to the targeted sites. Materials used in the fabrication of microspheres are nowadays biodegradable polymers, since surgical removal of delivery devices is not preferred. Several methods have been developed to prepare biodegradable microspheres. The preparation techniques have effects on the properties of microspheres and thus different techniques should be selected for different purposes. Techniques that have been used for microsphere preparation include emulsion, spray drying, phase separation, microfluidic preparation, self-assembly of supramolecules, and various polymerization techniques. The most popular current methods for preparing protein-loaded microspheres are the emulsion-based method and spray drying.

To encapsulate hydrophilic proteins, one step emulsion water-in-oil (w/o), or double emulsion water-in-oil-in-water (w/o/w) techniques are often used. In the w/o emulsion process [50, 51], aqueous protein/polymer solution is dispersed in a continuous organic phase containing oil-soluble surfactants, while the emulsion solution is being intensively stirred, homogenized, or sonicated. The aqueous droplets that are formed in the continuous phase are solidified by adding a water soluble crosslinker for the polymer. In some cases, emulsion droplets are physically solidified by using heat. The solidified microgels or microspheres are washed by a solvent, detergent, or water to remove organic phase chemicals and surfactants. The formation of stable emulsion droplets is critical for producing microspheres. Using appropriate surfactants or emulsifiers helps to reduce the surface tension between the dispersed aqueous droplets and the hydrophobic continuous phase [52].

For the production of very small microspheres, microemulsion is required, meaning formation of thermodynamically stable micellar droplets in the micron or submicron size range [53]. This microemulsion is achieved by using a surfactant concentration that is above its critical micelle concentration to prevent coalescence of micelles [52-54]. Protein-loaded microspheres have been prepared by this technique. A recent study showed that nanogels of chemically modified HA with a mean diameter of 200 nm were prepared using w/o techniques. The HA nanogels were used to entrap RNA, and were transported into cells successfully [55]. However, the spherical shape of these nanogels was not determined. In an earlier study, alginate microspheres were prepared by dispersing alginate solution in *n*-octanol containing hydrogenated

casteroil 60, and protein entrapping alginate microspheres with mean diameter of less than 5 μm were produced [56].

The limitation of the water-in-oil technique is that only highly water soluble polymers can be used. In order to use hydrophilic polymers, w/o/w methods have to be employed to encapsulate proteins. In this method, a hydrophilic protein solution is dispersed in a polymer dissolved in organic solvent to create a w/o emulsion, and then this primary emulsion is dispersed into a large volume of aqueous solution containing water soluble surfactants [57]. The organic solvent can be removed by heat, vacuum, or solvent extraction. This method allows the use of various synthetic polymers for protein encapsulation, which have more potential for the introduction of chemical modifications.

One possible problem for the w/o/w technique is the danger of denaturation of proteins, since the protein solution is directly dispersed in organic solvents. A copolymer of PCL and PLGA was used to prepare BSA-loaded microspheres with reasonable high encapsulation efficiency [58]. In this study, it was shown that protein release kinetics had a high dependence on the porosity of the microsphere surface, the internal structure of the microspheres, and the protein distribution inside the microsphere matrices. Emulsion techniques were also used to prepare PLGA, PLLA and PCL nanospheres with diameters ranging from 80 to 150 nm [59]. The results from this study showed that the polymer type and the surfactant amount and type all have effects on the size and size distribution of PLGA, PLLA and PCL nanospheres. In all emulsion techniques for preparing protein-loaded microspheres, the post-emulsion process to remove surfactant and organic residues by washing, heating, or solvent extraction could potentially damage or extract entrapped or encapsulated proteins from the porous microspheres. Nevertheless, one study suggested that the native confirmation of proteins could be stabilized by adding stabilizing excipients during the mixing of proteins and organic solvents in double emulsion techniques [60]. Another drawback of emulsion techniques is that of protein leakage during the dynamic emulsion process, which causes low encapsulation efficiency. The most important disadvantage is that the emulsion technique has a large reliance on the polymer properties and preparation parameters, which limits the number of polymers that can be used. Cautious investigation of these formulation parameters is needed.

The spray drying technique involves atomization of a dispersion of protein and polymer in a stream of hot air. Lyophilized protein is evenly suspended in organic polymer solution, and then the solution is atomized to small droplets in heated air. The small droplets containing proteins and polymers are solidified by quick solvent evaporation, followed by removal of organic solvent residues under vacuum. When hydrophilic protein/polymer is used, the aqueous solution is pre-mixed with crosslinker, and a fine mist of this solution is created by the atomizer. Typically, the diameters of microspheres formed by this method are in the micron and submicron range, depending upon the atomizing conditions. The particle size and size distribution can be controlled in this type of technique, and the results are highly reproducible. Chitosan has been a popular aqueous polymer used to prepare spray dried microspheres due to its fast ionic gelation property. Chitosan microspheres with diameters ranging from 6-9 μm were produced, and variation of the degree of crosslinking of chitosan was able to change the sphericity of the resultant particles [61]. Smaller chitosan microspheres ranging from 2-6 μm were fabricated by varying the preparation parameters [62]. In another study, insulin-encapsulating chitosan nanoparticles were prepared first by ionic gelation, then subsequently suspended in mannitol solution to form microspheres by the spray drying method. These nanoparticle aggregated chitosan microspheres had a smooth surface and spherical shape, with mean diameters from 3-4 μm [63]. In one earlier study, recombinant human erythropoietin was encapsulated in PLG microspheres prepared either by spray drying or by the double emulsion technique [64]. This study suggested that spray dried PLG microspheres had a smaller size, yielded higher protein encapsulation efficiency, and contained less organic solvent residue compared to double emulsion methods. It has been demonstrated that microspheres produced by the spray drying method are small in size and narrow in size range. However, the heated air and drying process used in spray drying still impose a hazard on proteins, since proteins are likely to aggregate and denature in high temperature.

The protein release mechanism of biodegradable microspheres is mainly dependent on polymer degradation, diffusion of proteins, and complexation between proteins and polymer matrices. Therefore, the rate of protein release could be regulated by chemically and physically engineering the polymer degradation rate, the permeability of the microspheres, the internal structure of microsphere matrices, and the interaction between proteins and matrix polymers. During release time, proteins at or near the surface of microspheres are initially released,

followed by slower release of proteins from the bulk or the core of microspheres depending upon the diffusion rate and distribution of proteins, and erosion and degradation of the microsphere core.

A commonly observed problem with protein release from microspheres is the initial burst release, which is principally due to the rapid diffusion of proteins from the surface of microspheres constructed from both synthetic and natural polymers [65, 66]. This initial burst effect can be reduced by several methods. In one study, the initial burst release of nerve growth factor (NGF) protein from PLGA microspheres prepared by spray drying methods was reduced to about 1% and the NGF was released for a period of 14 days [67]. The sustained release of NGF was achieved by adding zinc acetate to form a complex with NGF, which stabilizes the protein structures, and by adding zinc carbonate to PLGA solution prior to microsphere preparation, which slows down the hydration rate of microspheres and closes the surface pores of microspheres due to swelling. Similarly, the release profile of encapsulated insulin-like growth factor-I protein from PLGA microspheres was changed from a pattern of high initial burst followed by a slow release to one of low initial burst followed by a rapid release, since the protein distribution and hydrophobicity of PLGA were changed [68]. Increasing the protein-PLGA interactions by using additives and physically altering PLGA properties to reduce porosity of microspheres can also suppress initial burst effect and prolong the release time [69]. In the case of hydrophilic polymers, such as alginate and chitosan, modifying the microsphere surface and changing polymer compositions can help to reduce the initial burst effects. Alginate microspheres ionically coated with chitosan and polyethylene glycol were further covalently coated with chitosan and cellulose acetate phthalate, and initial release of heparin from the double coated alginate microspheres was moderately reduced as compared to microspheres with a single layer coating [70]. In another study, a decreased burst effect and sustained release of proteins from alginate beads was achieved by triply coating beads with high concentrations of polycations, polylysine or poly(vinyl amine) [71]. Coating BSA-loaded chitosan microspheres with hydrophobic polymers, such as paraffin oil and PLA, could decrease the porosity of microsphere surface, and thus lower protein initial release and increased release time [72].

Using appropriate microspheres as delivery devices can help stabilize the incorporated proteins, provide localized delivery, and achieve sustained release. However, there are some

challenges in preparing microspheres for optimal delivery of the proteins that are incorporated within them. Proteins as macromolecules have special three-dimensional structures, which directly affect the functions that they serve. Thus, maintaining the innate secondary, tertiary and quaternary structures of proteins during preparation and delivery process is extremely important for them to serve their biological functions. Since proteins can be digested by enzymes produced by the host, it is crucial to prevent proteins from unnecessary degradation until they reach their intended site of action. Therefore, mild process and formulation conditions are needed during protein encapsulation to avoid any loss of bioactivity. Another difficulty is to control the released dose at different periods over the time span of release. To solve this problem, the mechanism of protein release from different type of microspheres should be investigated thoroughly from chemical and physical points of view. Although the protein release kinetics may not be completely regulated, further research should be done to better understand and predict the release behaviours. To obtain the desired protein release profiles, the nature of polymer, polymer compositions, chemistry of crosslinking, properties of proteins, size of microspheres, and the surface and core properties of microsphere matrices should all be considered in designing protein release microspheres.

1.4. Alginate microspheres in tissue engineering

Alginate is a naturally occurring polymer derived from brown algae. The chemical structure of alginate is a linear unbranched polysaccharide containing alternating residues of 1,4'-linked β-D-mannuronic aicd (M) and α-L-guluronic acid (G) [73]. The molecular weight and chemical constitution of alginate depend on the source from which the alginate is isolated [74, 75]. The proportion and sequence of M and G residues determine the properties of alginate.

Alginate as a water soluble polymer can be gelled rapidly by using divalent cations except Mg^{2+}. Cations commonly used to form alginate gels are Ca^{2+}, Sr^{2+}, or Ba^{2+}. Although Ca^{2+} does not have the highest affinity toward alginate [76], it is commonly used due to its non-toxicity and low cost. The water soluble alginate is in its sodium salt form, where the Na^{+} is ionically bonded to G blocks of alginate. During the gelation process of alginate, divalent cations replace the Na^{+} associated with carboxyl groups on G blocks and fill in the interchain cavities, consequently one alginate chain dimerizes with other chains in alternation to form a complex gel network [73]. The calcium induced gelation of alginate has been studied. The ionic bonding

between G blocks of one chain and Ca^{2+} leads to the formation of interchain junction zones, which result in a stable and insoluble gel of alginate [77, 78]. Since Ca^{2+} only form bonds with G blocks, the proportion and the sequence of G blocks in alginate affect the properties of gels formed [73, 79].

Alginate gels have been tested for their biocompatibility. It has been reported that implanted alginate gels induced fibrosis in tissues [80], but other *in vivo* studies have shown that alginate does not provoke an inflammatory response [81]. In some cases, the impurity of alginate was found to be responsible for the inflammatory response and antigenic behaviour of alginate implants [81, 82]. Also, alginate polymer was shown to have mucoadhesion properties, which makes alginate a good candidate for drugs or proteins needed to be delivered via mucosal tissues [83]. However, the cell adhesion properties of alginate are poor; thus it does not promote cell attachment [42].

Alginate beads formed by a calcium-induced gelation process have been widely used for tissue engineering applications, such as cell encapsulation, and nucleic acid, drug, and protein delivery. Large alginate beads can be produced by dropping alginate solution into calcium chloride ($CaCl_2$) solution [84, 85]. The size of alginate beads formed by this method depends on the viscosity of the alginate solution, the size of the syringe, the distance between the needle and the surface of the $CaCl_2$ solution, and the flow rate of alginate solution extrusion [86]. Alginate beads prepared by this method normally have diameters greater than 1 mm. This mild technique for large bead formation is particularly suitable for encapsulating living cells in alginate hydrogel [87, 88].

Alternatively, alginate microbeads or microspheres used for protein encapsulation are commonly prepared by spray drying or emulsion techniques. The spray drying technique produces alginate microspheres by atomizing alginate solution to fine spherical droplets, which are further crosslinked by calcium ions [89-91]. As mentioned before, the emulsion technique involves dispersing alginate solution into an organic solution containing appropriate surfactants, followed by gelation or solidification process. The alginate micelles can be solidified internally or externally by using either calcium carbonate ($CaCO_3$) or $CaCl_2$ [92-96]. In the external gelation approach, the $CaCl_2$ solution is slowly dropped into the emulsion after the formation of alginate droplets in the emulsion. In the case of internal gelation method, fine grains or

microcrystals of $CaCO_3$ are evenly mixed with alginate solution prior to the emulsion processes, and then the solid $CaCO_3$ distributed in alginate droplets is liquefied by adding an oil soluble acid, typically acetic acid.

Due to the intrinsic differences in the gelation processes, the properties of alginate microspheres produced by each method vary. Alginate microspheres produced by the internal gelation method have homogenous internal structure but a porous surface, while the external gelation method provides compact surface structure but an unevenly distributed internal polymer network. The size and size distribution of alginate microspheres prepared by the emulsion method also vary depending upon the preparation parameters, such as the type of organic phase and surfactant and stirring speed. In a previous study, alginate solution was dispersed into silicone oil to prepare alginate microspheres by using the internal gelation method [97]. The average diameters of alginate microspheres were 489±32 μm and ranged from 120 to 1600 μm. Another earlier study used iso-octane containing Span 85 as the continuous organic phase to disperse alginate solution, and microspheres were gelled externally. The alginate microspheres prepared this way had spherical shapes and smooth surfaces, with diameters smaller than 150 μm but larger than 10 μm. A later study investigated the effects of different surfactants in iso-octane on the sizes of resultant alginate microspheres [66]. Alginate microspheres prepared using a high viscosity solution have diameters ranging from 1 to 150 μm. It was also found that using the iso-octane/Span 85 emulsion formulation could make alginate microspheres with a mean diameter of 7.6±0.1 μm for low viscosity alginate [66]. An attempt to produce small alginate microspheres used *n*-octanol as the organic phase and HCO-60 as a surfactant [56]. The resultant alginate microspheres had smooth surface and the diameters ranged from 0.5 to 5 μm.

All calcium induced alginate gelation undergoes the same chemical reaction, which is the formation of an alginate network by crosslinking of G blocks. Thus, the mechanism of calcium-alginate hydrogel biodegradation is the same, but the rate is different depending upon the chemical composition of alginate and the structure of the polymer network. Since mammals do not produce enzymes specifically for alginate, the degradation of alginate hydrogels is not enzymatic. The mechanism of alginate degradation is a hydrolysis or dissolution process, where crosslinking calcium ions are released from gels into the surrounding media. The large alginate chains remaining after the dissolution process are unlikely to be removed completely by the host,

because the molecular weights of commercial alginate are higher than the renal clearance threshold of the kidneys [98]. Both *in vivo* and *in vitro* studies have demonstrated that the calcium crosslinked unmodified alginate hydrogels degrades through ionic exchange of crosslinking calcium ions with monovalent ions from the media at neutral pH, which is an uncontrollable process [99, 100]. The uncontrollable exchange rate of calcium and monovalent ions could be problematic for regulating the release rate of encapsulants. The release rate of protein from alginate microspheres is mainly dependent on the dissolution rate of alginate and the diffusion rate of proteins. The dissolution rate of unmodified calcium-alginate gels, as mentioned, is difficult to control, but the degradation rate of chemically modified alginate can be controlled [99]. Also, the release of protein from alginate gels could be rapid because of the hydrophilic nature of alginate and the porous feature of hydrogels [101]. However, the diffusion rate of proteins could be reduced by increasing protein-gel interaction [70], coating gels to reduce permeability of microsphere surfaces [102], or regulating the internal gel structures [103].

Alginate microspheres or nanospheres have attracted considerable attention as potential protein delivery systems. Calcium-alginate gels can be produced using relatively mild conditions, suitable for encapsulating and protecting proteins. An earlier study used alginate microspheres encapsulating nerve growth factor (NGF), fabricated using the single emulsion method, to treat degeneration of cholinergic neurons by implanting microspheres in the lesioned pia arachnoid vessels in the central nervous system of rats [104]. The immunocytochemical results of *in vivo* release studies showed that sustained release of NGF can prevent neuronal degeneration, and the microsphere delivery systems were comparable with installed cannulae. The protein release time can be extended by improving the protein-alginate interaction. An *in vitro* study encapsulated proteins with high isoelectric point (p*I*) into alginate microspheres and compared release profiles of high and low p*I* proteins [105]. The release of high p*I* protein was shown to be more prolonged than that of low p*I* protein from alginate microspheres due to the electric bonding between high p*I* proteins and alginate polymers [105]. Heparin binding vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have been encapsulated into alginate microgels, and sustained releases of both proteins were observed *in vitro* [16]. These growth factor-loaded alginate gels were tested *in vivo*, and it was found that both growth factors were delivered subcutaneously and promoted angiogenesis in severe combined immunodeficient mice. In another study [106], bFGF protein was loaded into already formed heparin-alginate

microbeads by immersion, with an 80% encapsulation efficiency. The bFGF protein was delivered through these heparin-alginate microcapsules implanted in ischemic but viable unrevascularizable myocardium in patients undergoing coronary artery bypass graft surgery, and the results showed that using these microcapsules to deliver bFGF was a safe and feasible method.

The poor cell adhesion property of alginate can be improved by coating alginate microspheres with cell adhesive proteins. Peptide-loaded alginate microspheres produced by the emulsion technique were covalently coated with human serum albumin [107]. This *in vitro* study using osteoblast cultures demonstrated that the protein coating reduced the release rate of peptides from alginate microspheres, and increased their cell adhesive properties.

Sustained release can also be achieved by coating alginate microspheres with chitosan [71, 108-110]. The chitosan membrane around alginate microspheres decreases the swelling rate and gel erosion, and thus delays the protein diffusion throughout the gels. It was reported that sustained protein release can be achieved from chitosan coated alginate microspheres prepared with alginate with high molecular weight and high G unit contents, and chitosan with high molecular weight, large charge density, and appropriate coating concentrations [71, 108, 109, 111, 112]. Alginate microspheres also have been coated with silk fibroin to improve mechanical stability and to prolong the time span of release [113], or with polylysine to form a diffusion barrier to encapsulants and to protect microspheres from hydrolytic enzymes [114, 115].

Since the size, size distribution, morphology, and swelling features of alginate microspheres have direct influences on the rate and time span of protein delivery, it is worthwhile to investigate how the preparation parameters used in the emulsion technique can change the physical properties of alginate microspheres. The emulsion technique has been demonstrated to create microspheres with good morphology. The emulsion system using liquid paraffin oil as a continuous phase and 0.5-4% of Span 80 as a surfactant was used to prepare chitosan microspheres. The smallest mean diameters were 297 μm when a stirring speed of 700 rpm was used [116]. When the stirring speed was increased to 3000 rpm, the diameter of the chitosan microspheres produced by using 1% Span 80 in paraffin oil as continuous phase was 10.2±0.21 μm [117]. It seems worthwhile to test the potential of the paraffin oil/span 80 emulsion system to produce alginate microspheres in 50 mL of paraffin oil, since the paraffin

oil/Span 80 emulsion volume used in the past cases was large than 50 mL. Also, the factors that influence the size of alginate microspheres in this emulsion system need to be investigated. The feasibility of ionically crosslinking alginate microspheres using externally supplied $CaCl_2$ during the emulsion process need to be studied.

Since BSA has been used as a common small protein model in past studies, the use of BSA could help to establish the loading and release behaviour of alginate microspheres prepared using different methods. A few attempts have been made to create microsphere-incorporated scaffolds as a protein delivery device in order to extend and control the protein release [118, 119]. Therefore, the BSA-loaded alginate microspheres created by the paraffin oil/Span 80 emulsion system need to be studied to optimize the synthesis conditions. Also, BSA release profiles from microspheres embedded within alginate scaffolds need to be investigated. The results obtained from BSA-loaded alginate microspheres can be used to determine their functions as protein release system, and the release behaviours of microsphere-embedded alginate scaffolds can be used to assess their ability as a double release system. However, the use of large emulsion volume could not meet the practical needs of producing small quantity of microspheres with small amount of protein, and the use of BSA could not satisfy the need of establishing a release model for larger proteins. Since the effective dose of many large proteins is in the nanogram range, a procedure to encapsulate smaller amounts of large molecular weight protein in alginate microspheres need to be built. Based on the results obtained from BSA-loaded alginate microspheres, the emulsion/external gelation process needs to be refined to meet practical laboratory needs. Also, the total volume of emulsion mixture and the protein loading percentage need to be reduced. A larger protein, immunoglobulin G (IgG), was encapsulated in alginate beads by extruding alginate/IgG solution into $CaCl_2$ solution in a previous study, but it was not commonly used as a protein release model to study microspheres prepared using the emulsion technique. It is also attractive to study the loading and release behaviours of IgG due to its unique quaternary structure. The release profiles of IgG from alginate microspheres produced in an optimized process and from scaffolds loaded with different amount of IgG-microspheres could provide helpful information for continuing future work on using such a system to deliver large proteins in a controlled manner *in vivo*.

1.5. Research objectives

The aim of this research is to prepare alginate microspheres using an emulsion technique and investigate their potential as a protein delivery system. This study serves the goal of fabricating microparticles for sustained delivery of bioactive macromolecules as part of a tissue engineering strategy to improve tissue regeneration.

Objectives:

1. Examine the feasibility of using the emulsion technique to produce alginate microspheres.
2. Study the ability to control microsphere size and size distribution by varying emulsion process parameters.
3. Compare loading and encapsulation efficiency using incubation and incorporation methods of protein loading.
4. Measure the release profile of two model proteins from alginate microspheres and analyze parameters that influence release rate.
    a. Protein loading methods
    b. $CaCl_2$ concentration
    c. Chitosan coating
    d. Protein properties
    e. Release media
5. Study the feasibility of embedding alginate microspheres into a large 3D alginate cylindrical scaffold matrix
6. Investigate the release profiles of model proteins from microsphere-incorporated alginate cylindrical scaffolds and analyze influences of preparation parameters on protein release rate.
    a. Crosslinking process of alginate scaffolds
    b. Microsphere densities within scaffolds

1.6. Thesis organization

This thesis consists of three major components, which are an introduction, description of the experimental preparation and characterization of protein loaded alginate microspheres and scaffolds, and comparative discussion and conclusions.

The second chapter of the thesis presents the development and characterization of BSA-loaded alginate microspheres and microsphere-incorporated alginate scaffolds. The use of the emulsion technique to create alginate microspheres was examined first. The effects of different preparation parameters on the morphology, size, and release profiles of microspheres were also examined. The profiles of release of BSA from alginate microspheres embedded in alginate scaffolds were measured, and the influences of microsphere densities in alginate scaffolds were studied.

The third chapter demonstrates a scaled down preparation method for IgG-loaded alginate microspheres, which is more suitable for delivery of protein in microgram and nanogram scale. The feasibility of producing small alginate microspheres using different parameters in emulsion technique was examined. The release profiles of IgG from small alginate microspheres and the effects of chitosan coating on the release rate were analyzed. The release of IgG from small alginate microspheres embedded in alginate scaffolds was also investigated.

A detailed and comparative discussion of results obtained from chapter 2 and 3 and conclusions are presented in chapter 4. The different parameters used in emulsion processes for producing alginate microspheres containing BSA and IgG were compared, and the factors influencing the morphology and size of alginate microspheres were addressed. The release profiles of two model proteins were compared and the parameters that influence the release rate were analyzed. The effects of different conditions used in release studies on release profiles of two models proteins were evaluated and the practical aspects of these conditions were discussed. Also, the use of microspheres incorporated within alginate scaffolds as a protein release device was assessed.

The last chapter summarizes this research and recommends potential future work.

Chapter 2. Alginate microspheres for release of BSA

2.1. Introduction

Alginate microspheres have been widely used as delivery vehicles for macromolecules *in vitro* and *in vivo* due to their biocompatibility and non-toxicity [71, 87, 102, 120-122]. One common technique, water-in-oil emulsion [56, 95], is able to create microspheres at micro-level. The protein loaded in alginate microspheres is released at the destination site due to the degradation or erosion of microspheres. Therefore, the protein release kinetics is highly dependent on the degradation properties of alginate gels. In order to accomplish sustained protein release, coating microspheres with other material(s) can create a diffusion barrier to ion flux, thereby preserving ionic crosslinking [71, 110, 112]. In tissue engineering, the goal of delivering proteins is to provide proteins at a desired location in a controlled manner. The release of proteins could be retarded and localized using scaffolds containing protein encapsulated microspheres. In fact, microspheres incorporated into scaffolds can be used as polymeric delivery vehicles to deliver functional protein over a long period of time [123-126]. Microsphere incorporated polymeric scaffolds have the advantages of reducing the osmotic burst effect, increasing the local protein concentration, and controlling protein release rates, compared to protein-loaded porous microspheres alone [126, 127]. It has been found that the chemical and physical properties of microsphere and scaffolds influence the protein release kinetics [128]. Protein release is controlled by the diffusion-erosion process of microspheres and hydrogels, suggesting the release rate might be effectively controlled by optimizing the preparation parameters of microspheres and hydrogels.

In the work presented in this chapter, alginate was used to synthesize alginate microspheres using the paraffin oil/Span 80 emulsion system, and BSA was selected as a model protein and loaded into alginate microspheres. The aim of the first part of the research is to construct a procedure for preparing BSA-loaded alginate microspheres using the chosen emulsion system. The release profile of protein from microspheres was examined *in vitro*, with a focus on the influence on the release profile of such factors as the crosslinker concentration, protein loading methods, and polymeric coating of microspheres. In addition, cylindrical alginate scaffolds were prepared with incorporated microspheres, and protein release profiles from different types of scaffold construct were also examined.

2.2. Materials and methods

2.2.1. Materials

Low-viscosity alginic acid sodium salt (A2158), middle-viscous chitosan, calcium chloride ($CaCl_2$), bovine serum albumin (BSA), paraffin oil, and Span 80 were purchased from Sigma-Aldrich (Oakville, Canada). Alginate (A2158) has a mannuronic to guluronic acid ratio of 1.67 and an average molecular weight of 50 kDa [129]. Dulbecco's modified Eagle's medium (DMEM) was obtained from Invitrogen (Burlington, Canada).

2.2.2. Preparation of alginate microspheres

Sodium alginate was dissolved in water to obtain a final alginate concentration of 5% (wt/vol). Microspheres were prepared by means of the modified emulsification and internal gelation methods [92, 120]. Briefly, the 5% alginate solution was dispersed into paraffin oil containing 5% Span 80 with a ratio of 1:4 (i.e., the volume of aqueous phase / the volume of oil phase); by stirring at 1200 rpm using a mechanical stirrer, and the emulsification process was continued for 30 minutes at room temperature. Then, either 1% or 10% (wt/vol) of $CaCl_2$ solution was added slowly into the emulsion mixture. The mixture was then continuously stirred at 1200 rpm for two hours to permit gelation of microspheres with calcium cations, which was followed by the addition of 10 mL of 100% isopropyl alcohol to harden the microspheres that were formed. After stirring for 10 minutes, the microspheres were collected by centrifugation at an acceleration of 200 g for 10 minutes at room temperature. Subsequently, collected microspheres were washed twice with isopropyl alcohol and water, and lyophilized for 24 hours in a freeze dryer (Labconco Co.). Alginate microspheres were prepared in triplicate for the sequential tests.

2.2.3. Preparation of chitosan-coated alginate microspheres

Chitosan was dissolved in 2% acetic acid to a concentration of 0.1%, the pH value of the obtained chitosan solution was adjusted to 5 by adding 1M NaOH. Freeze-dried alginate microspheres prepared according to the above methods were dispersed into 0.1% chitosan solution containing 5 mM $CaCl_2$, and were then gently shaken for 1 hour at room temperature to allow adsorption of cationic chitosan molecules onto the anionic surface of alginate microspheres.

The chitosan-coated microspheres were collected by centrifugation, washed twice with water, and then lyophilized.

2.2.4. Physical characterization of alginate microspheres

The size of microspheres was measured using an optical microscope (Akioskop) equipped with a camera. The freeze-dried microspheres were suspended in water and then photographed immediately. The images of microspheres were analyzed by Northern Eclipse image analyzing software (Empix Imaging, Mississauga, ON). The diameter of microspheres was averaged from one hundred sample microspheres in each batch, and two batches from each condition were analyzed.

The shape and morphology of microspheres were also visualized by scanning electron microscopy (SEM). The freeze-dried microspheres were deposited on a conductive tab pressed on to a specimen holder and coated with gold under vacuum. The SEM photographs were obtained using a Philips 505 scanning electron microscope.

2.2.5. Preparation of BSA-loaded alginate microspheres

2.2.5.1. Incorporation method - loading BSA during emulsion

BSA protein were added into 5% alginate solution, and mixed for 10 minutes by using a mechanical stirrer. Subsequently, the alginate-BSA mixture was added to paraffin oil containing Span 80 and microspheres were synthesized as described previously.

2.2.5.2. Incubation method - loading BSA by immersion

In addition to the above incorporation method, an alternative protein encapsulation method is the incubation method [105, 130]. Specifically, the freeze-dried blank microspheres were suspended in a solution containing 0.15% (wt/vol) of sodium chloride (NaCl) and known amounts of BSA. The mixture was then gently agitated for 2 hours at room temperature to allow uptake of BSA into microspheres, after which the microspheres were soaked in 1 M of $CaCl_2$ for 10 minutes. The microspheres were centrifuged at 1000 rpm for 10 minutes, and then washed twice with water. For every 10 mg of dried microspheres, 1 mL of 0.1 mg/mL or 1 mg/mL of BSA solution was used.

2.2.6. Determination of encapsulation efficiency

Microspheres were weighed by a scale, and then suspended in phosphate buffered saline (PBS). The samples were kept in PBS and shaken for 24 hours to promote release of all loaded protein. The samples were centrifuged at 4000 rpm for 15 minutes, and the protein content in the supernatant was analyzed using a Bradford protein assay with spectrophotometry at a wavelength of 595 nm. The blank microspheres were used as a control group. The encapsulation efficiency and loading efficiency were determined from the average of three batches of samples, and was calculated from the following equations, respectively.

$$\text{Encapsulation efficiency } (\%) = \left(\frac{\text{Mass of loaded protein}}{\text{Mass of added protein}}\right) \times 100 \qquad \text{(Equation 2.1)}$$

$$\text{Loading capacity } (\%) = \left(\frac{\text{Mass of loaded protein}}{\text{Mass of microspheres}}\right) \times 100 \qquad \text{(Equation 2.2)}$$

2.2.7. Studies of protein release from microspheres *in vitro*

For coated and uncoated BSA-loaded microspheres prepared under varying conditions as listed in Table 2.1, 20 mg of dried microspheres were suspended in 1 mL of 10 mM PBS (pH7.4) in a microcentrifuge tube. The samples were incubated at 37 °C while shaking at 100 rpm. At predetermined time intervals, the samples were centrifuged at 5000 rpm for 10 minutes, and the whole 1 mL of release medium was collected. The BSA concentration in the supernatant collected was determined using a Bradford protein assay. The pellets of microspheres were resuspended in 1 mL of fresh PBS and the procedure was repeated until the end of the experiment.

Table 2.1. Conditions used to prepare alginate and BSA-loaded alginate microspheres.

Abbreviations	$CaCl_2$ concentration	BSA loading method	Chitosan coating
1%-Alg-Ch	1%	No BSA	yes
1%-Alg-Un			no
1%-Incorp-Ch		Incorporation method	yes
1%-Incorp-Un			no
1%-Incoub-Ch		Incubation method	yes
1%-Incub-Un			no
10%-Alg-Ch	10%	No BSA	yes
10%-Alg-Un			no
10%-Incorp-Ch		Incorporation method	yes
10%-Incorp-Un			no
10%-Incub-Ch		Incubation method	yes
10%-Incub-Un			no

2.2.8. Preparation of microspheres embedded within alginate cylinder scaffolds

Accurately weighed dry microspheres were mixed with 2% alginate solution to obtain a weight-to-volume percentage of 5%. The microsphere-alginate mixture was immediately injected into a Silastic tubing (Dow Corning, Midland, MI) with an inner diameter of 1.47 mm. The tubing was immersed in 1 M of $CaCl_2$ overnight at 4 °C, and a cylindrical-shaped alginate scaffold was formed, which was then pushed out the tubing and soaked in 1 M of $CaCl_2$ at 4 °C for 2 hours before use.

2.2.9. Studies of protein release from scaffolds *in vitro*

The cylindrical microsphere-alginate scaffolds were cut into pieces with a length of 1 cm. Alginate cylinders incorporated with BSA-loaded-microsphere, as well as a control sample made with blank microspheres, were placed into 24-well cell culture plates. Each alginate cylinder was placed in one well, and 1 mL of DMEM was added as release medium. The plates were then agitated in a shaker at 100 rpm for 48 hours at 37 °C. At scheduled time intervals, 1 mL of release medium was collected and analyzed for BSA concentration. Release medium was replaced with 1 mL of fresh DMEM. All BSA concentration analyses were done in triplicate.

2.3. Results and discussion

2.3.1. Alginate microspheres

Figure 2.1, 2.2, and 2.3 are optical and SEM micrographs of alginate microspheres, chitosan coated alginate microspheres, and BSA-loaded microspheres. The images show that the paraffin oil/Span 80 emulsion system is able to produce alginate microspheres with good spherical shape and a smooth surface. From Figure 2.2 and 2.3, it is observed that the chitosan coated alginate microspheres have fuzzier surfaces and there are more conjunctions of spheres. The formation of a thin chitosan membrane surrounding the alginate microspheres contributes to the surface characteristics. Using the emulsion technique, alginate microspheres with or without BSA have been successfully prepared under the conditions listed in Table 2.1.

The particle size analyses showed that the concentration of $CaCl_2$ solution, and the chitosan coating have effects on the diameters of alginate microspheres (Table 2.2). The measurements of diameters revealed that particle size increases when the amount of calcium

crosslinker used decreases, and that the chitosan coating increases the diameters of microspheres as expected. Figure 2.3 shows the size distribution of alginate microspheres prepared with 10% $CaCl_2$. Increasing $CaCl_2$ concentration during the emulsion process reduces the diameters and narrows the size distribution of alginate microspheres. The majority of alginate microspheres are approximately 10 μm in diameter (Figure 2.4). The mean diameters of microspheres were reduced from 20.5±9.1 μm to 6.5±2.8 μm when the $CaCl_2$ concentration used in the gelation process was increased. The decrease in microsphere size and size distribution is mainly caused by the syneresis of alginate hydrogels in high calcium concentration [87]. The shrinkage of alginate microspheres volume occurs due to contractions of alginate polymers upon crosslinking reaction with calcium ions. The changes in the mean diameters revealed that the syneresis effect increases as the calcium concentration increases.

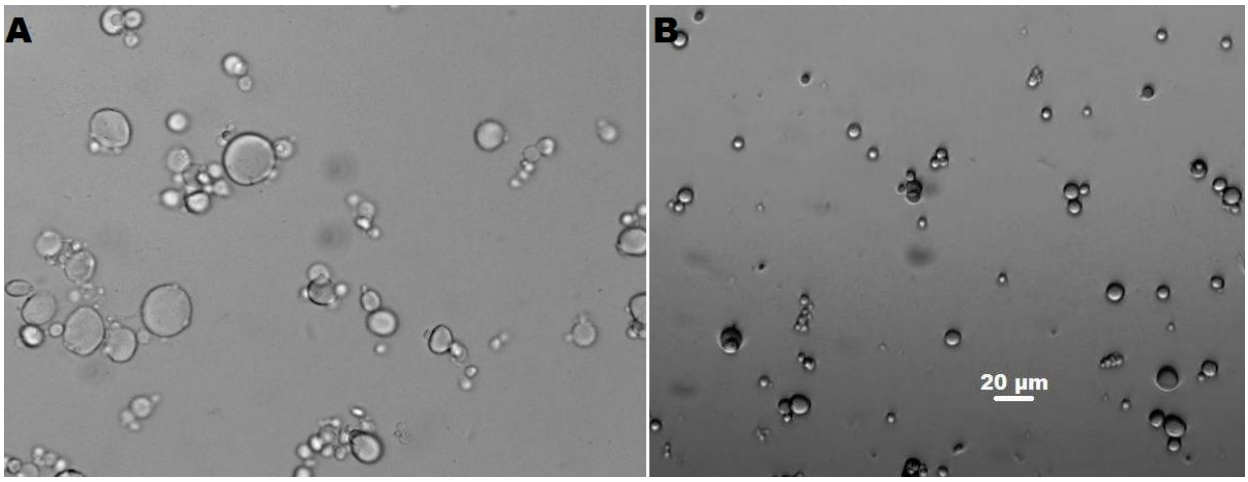

Figure 2.1. Optical photomicrograph of alginate microspheres prepared with (A) 1% $CaCl_2$ and (B) 10% $CaCl_2$, both of which have the same scale.

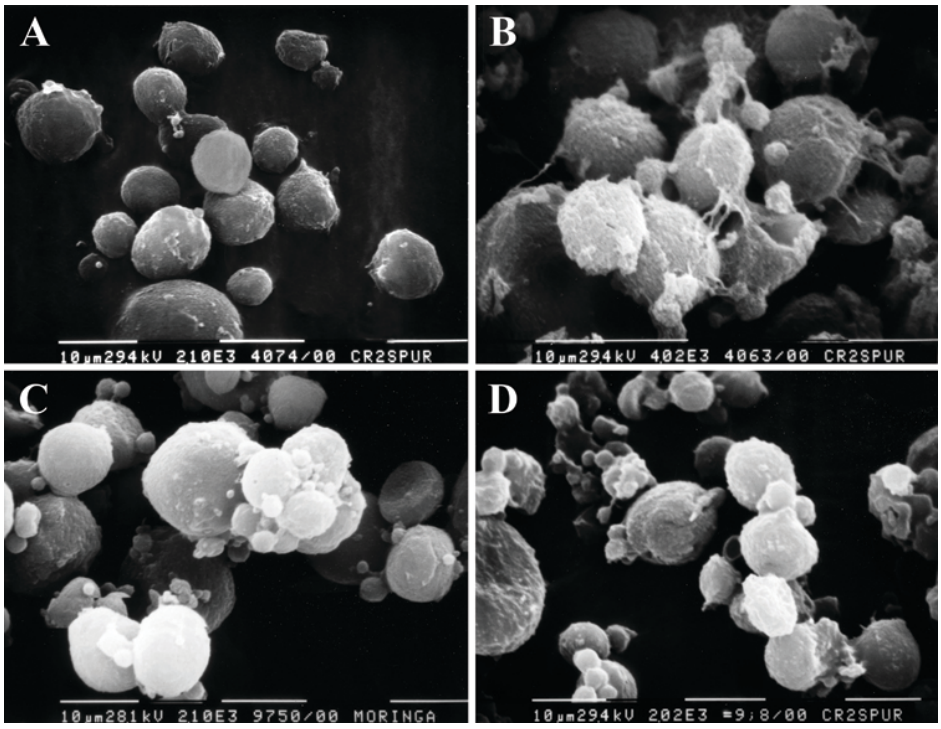

Figure 2.2. SEM micrographs of alginate microspheres prepared with 1% $CaCl_2$. (A) alginate microspheres, (B) chitosan coated alginate microspheres, (C) BSA loaded-alginate microspheres by incorporation methods, and (D) BSA loaded-alginate microspheres by incubation methods.

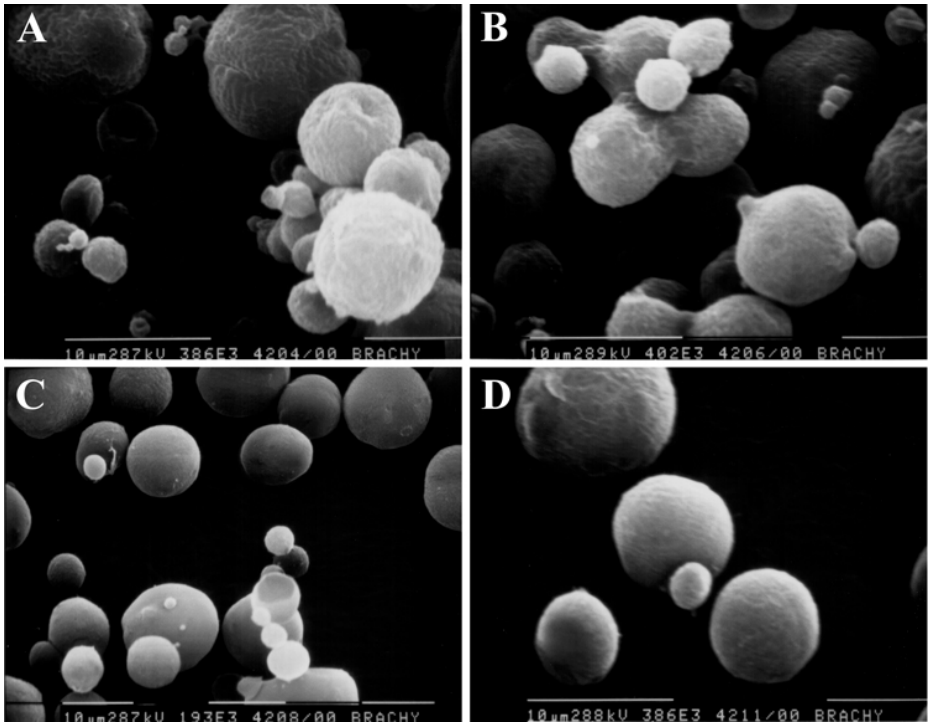

Figure 2.3. SEM micrographs of alginate microspheres prepared with 10% $CaCl_2$. (A) alginate microspheres, (B) chitosan coated alginate microspheres, (C) BSA loaded-alginate microspheres by incorporation methods, and (D) BSA loaded-alginate microspheres by incubation methods.

Table 2.2. Diameters of microspheres made under different conditions**.**

$CaCl_2$ Concentration used (weight/volume)	Types of microspheres	Mean Diameters (μm)
1%	Alginate microspheres	20.5 ± 9.1
	Alginate microspheres coated with chitosan	21.7 ± 9.3
	Alginate microspheres BSA loaded by incorporation method	23.1 ± 9.6
	Chitosan coated alginate microspheres BSA loaded by incorporation method	24.8 ± 10.2
	Alginate microspheres BSA loaded by incubation method	22.4 ± 10.7
	Chitosan coated alginate microspheres BSA loaded by incubation method	23.3 ± 11.5
10%	Alginate microspheres	6.2 ± 2.8
	Alginate microspheres coated with chitosan	7.6 ± 3.3
	Alginate microspheres BSA loaded by incorporation method	8.0 ± 3.6
	Chitosan coated alginate microspheres BSA loaded by incorporation method	10.1 ± 5.0
	Alginate microspheres BSA loaded by incubation method	8.2 ± 4.3
	Chitosan coated alginate microspheres BSA loaded by incubation method	9.0 ± 4.8

All measurements were made using freeze-dried microspheres suspended in water. Data are shown in mean ± S.D. (n = 200).

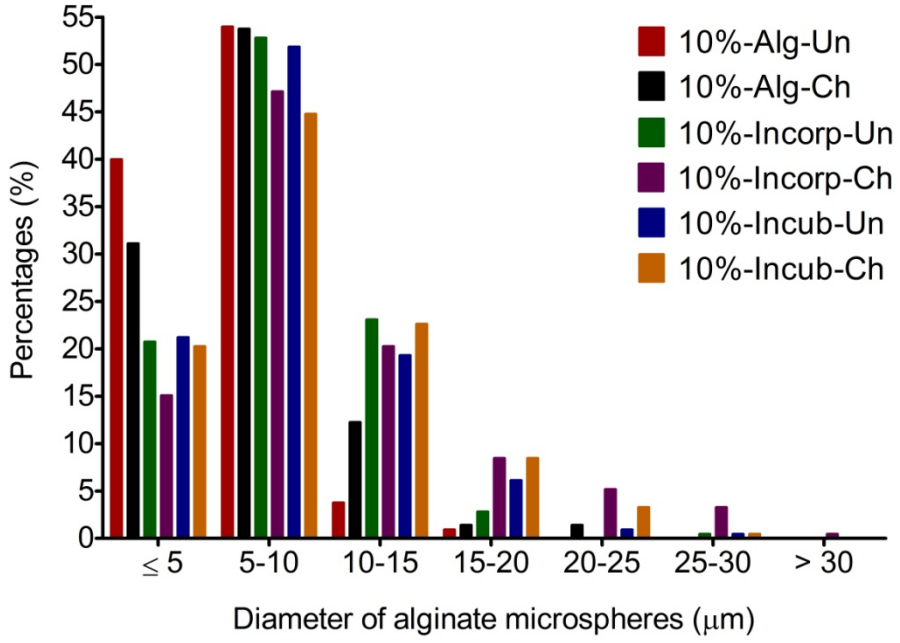

Figure 2.4. Size distribution of alginate microspheres made with 10% $CaCl_2$.

2.3.2. Encapsulation efficiency of different loading methods

The loading efficiency and encapsulation efficiency of BSA loaded alginate microspheres are calculated and reported in Table 2.3. The theoretical maximum loading efficiency is 1%, since the weight ratio of alginate and BSA used is 100 to 1. It was found that BSA loading efficiency is well below the theoretical maximum in all cases. However, the incorporation methods produced much better encapsulation efficiency than the incubation method. This could be explained by the electrostatic charges on BSA and alginate molecules at neutral pH. At neutral pH, the repulsive force between the negatively charged BSA and alginate contribute to the reduction in the encapsulation efficiency of the incubation method. The modest encapsulation efficiency of incorporation method could be affected by protein-alginate interaction during the emulsification process and by protein diffusion during washing steps after the microspheres have formed. The BSA molecule is known to have a small hydrodynamic volume and molecular weight [131], which increase the possibility of diffusing out of microspheres during the preparation processes. However, in the case the incubation methods, the small size of BSA does not offer significant advantages. The low encapsulation efficiencies of microspheres using incubation methods suggest that the negative charges of BSA are more influential than size in term of diffusive properties in alginate gels.

Table 2.3. Calculated loading efficiency and encapsulation efficiency of alginate microspheres.

$CaCl_2$ concentration	BSA loading method	Loading efficiency (% w/w)	Encapsulation efficiency (% w/w)
1%	Incorporation method	0.44	44
	Incubation method	0.04	4.5
10%	Incorporation method	0.36	39
	Incubation method	0.08	7.8

The shown loading and encapsulation efficiency are the averages of three percentages and are calculated using Equation 2.1 and 2.2, respectively.

2.3.3. Effects of preparation parameters

The *in vitro* BSA release from alginate microspheres prepared under different conditions was investigated under isothermal conditions. The influence of $CaCl_2$ concentration used during emulsion processes, the protein loading methods, and the chitosan coating on alginate microspheres on BSA release behaviours was studied in PBS at 37 °C.

Figure 2.5 shows that BSA loaded using incorporation methods was released more slowly than when loaded using incubation methods, despite the amount of crosslinking regents used. In all of the protein release experiments, protein loaded with incubation methods was released faster than incorporation methods during all stages of release. One possible cause is that, during incubation time, more proteins were deposited on the microsphere surface rather than diffused into microspheres. Since the protein release kinetics is affected by both protein diffusion kinetics and degradation of microspheres themselves, the proteins adhered onto the microsphere surface contribute largely to the faster release.

The influence of degradation of microspheres on release profiles was studied through altering the crosslinking degree of alginate microspheres by using different amount of $CaCl_2$; presumably, more calcium cations would provide a higher degree of crosslinking. For protein loaded with incorporation methods (Figure 2.6), increased calcium cations slowed down the protein release over the time course of the experiment. However, for protein loaded with incubation methods, superimposed release profiles were observed for microspheres prepared with 1% and 10% $CaCl_2$. This observation also could be explained by proteins adhering onto the microsphere surface during incubation loading. Therefore, the degree of crosslinking of microspheres did not play an important role in protein release rate from incubation-loaded microspheres, since a large portion of the protein was simply dissociated from the microsphere surface instead of diffusing from inside. For microspheres prepared using the incorporation method, higher concentration of calcium cations delayed the protein release slightly during the first 5 hours of releasing time course, but after the initial bursting period, incorporation-loaded microspheres made with low and high concentration of calcium cations showed similar release profiles.

The dissociation and diffusion of proteins from the surface and from the inside of alginate microspheres was delayed by coating alginate microspheres with diluted chitosan solution in all tested cases. A better control of BSA release was observed in chitosan coated microspheres (Figure 2.5 and 2.6). Not surprisingly, chitosan coated alginate microspheres made with either 1% or 10% $CaCl_2$ showed slower and more sustained release of proteins comparing to uncoated ones, from which proteins were completely released in the first 5 hours. In the case of chitosan coated microspheres, protein release was still detectable at 48 hours. It has been established that chitosan coating stabilizes alginate microspheres and lengthens the protein release [71, 110, 112, 132]. The positively charged amino groups of chitosan form ionic bonds with negatively charged carboxyl groups of alginate, forming a thin layer of chitosan membrane on alginate microspheres. The chitosan layers protect alginate microspheres from osmotic pressure and diffusion of ions, and thus reduce the initial burst release and rate of decrosslinking of microspheres.

The initial burst effect of alginate microspheres is inevitable in all cases. However, higher degree of crosslinking of alginate polymer can slow down the burst effect to an extent. Also, the complete release of proteins could be delayed from 5 hours to 50 hours by coating the alginate microspheres. Alginate microspheres loaded with incubation methods presented faster and shorter release profiles in comparison to ones loaded with incorporation methods.

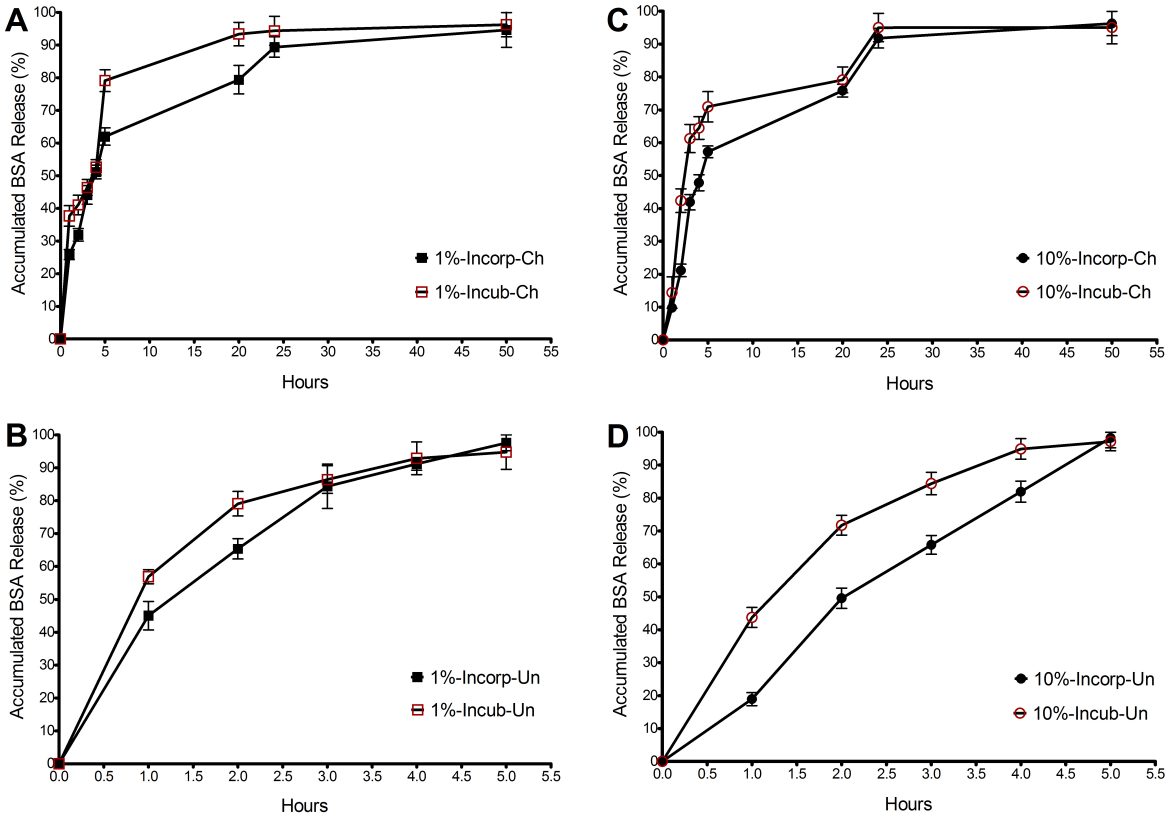

Figure 2.5. The effect of protein loading methods. In each panel, the accumulated BSA release of alginate microspheres prepared with either incorporation or incubation method in PBS are represented as mean ± S.D. (n = 3). Chitosan coated alginate microspheres prepared with either 1% $CaCl_2$ (A) or 10% $CaCl_2$ (C). Uncoated alginate microspheres prepared with either 1% $CaCl_2$ (B) or 10% $CaCl_2$ (D).

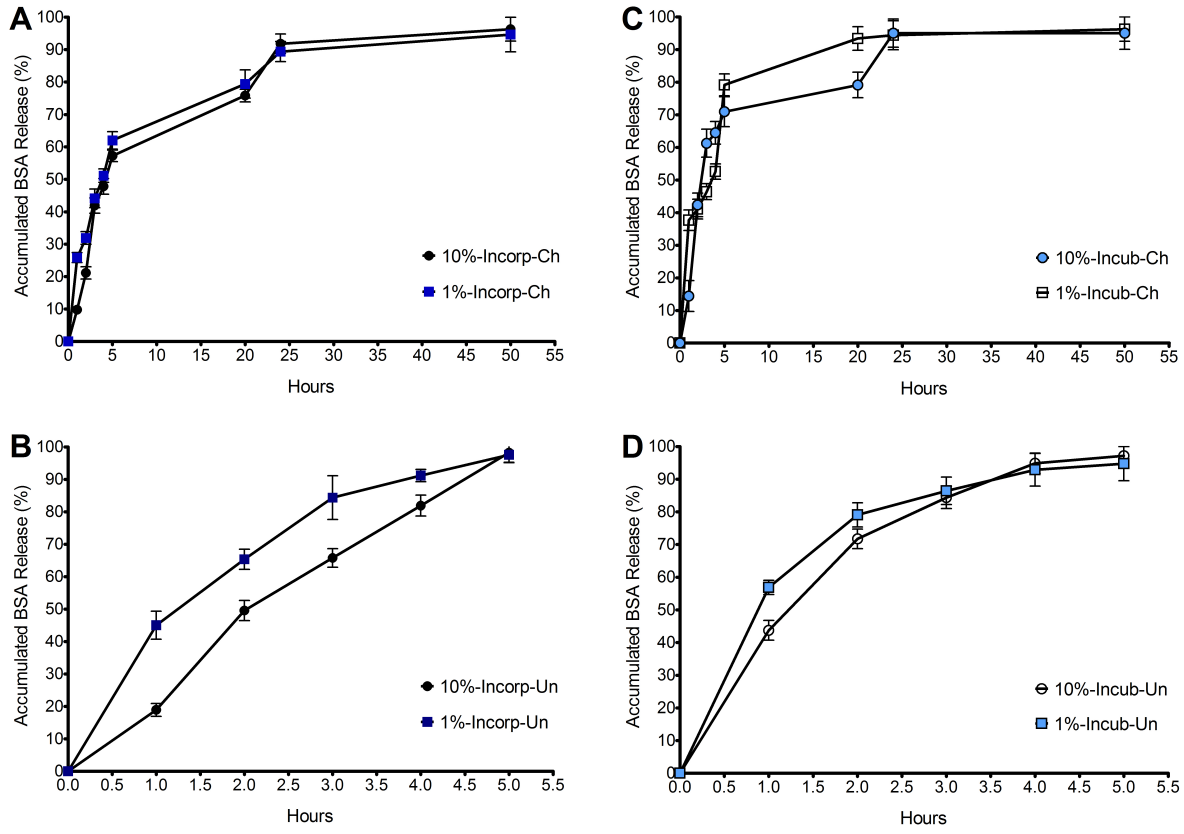

Figure 2.6. The effect of $CaCl_2$ concentration. In each panel, the accumulated BSA release in PBS of alginate microspheres prepared with either 1% or 10% $CaCl_2$ are represented as mean ± S.D. (n = 3). Chitosan coated alginate microspheres loaded with incorporation method (A) or incubation method (C). Uncoated alginate microspheres loaded with incorporation method (B) or incubation method (D).

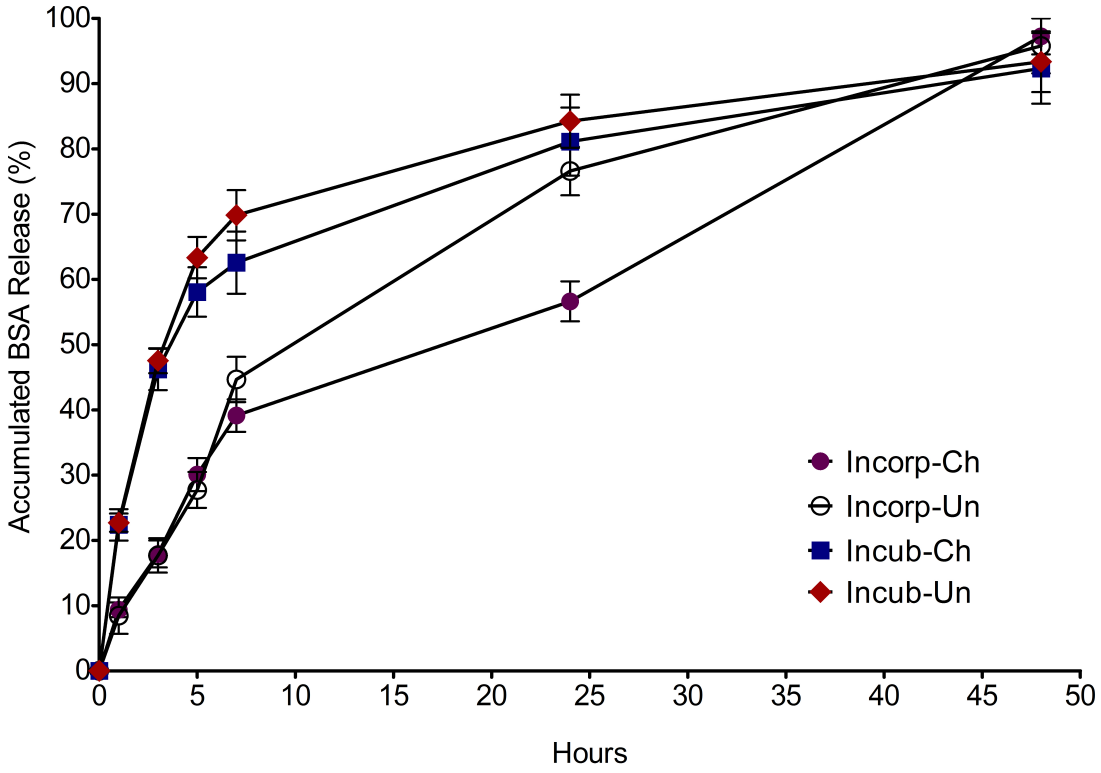

Figure 2.7. Release profiles of BSA from microsphere-incorporated alginate scaffolds in DMEM at 37 °C. Alginate scaffolds prepared using 10%-microspheres with: coating and incorporation method (●), uncoated and incorporation method (○), coating and incubation method (■), and uncoated and incubation method (♦). Values represent means ± S.D. (*n* = 3).

2.3.4. Studies of microsphere-containing alginate scaffolds *in vitro*

Alginate cylindrical scaffolds containing incorporated microspheres were moulded in silicone tubing overnight, and further gelled in $CaCl_2$ for 2 hours after solid gels were obtained. The external gelation process was purely dependent on the diffusion of calcium ions into alginate hydrogels. Shrinkage in hydrogel volume was observed; thus, the diameters of gelled alginate scaffolds were approximately 80% of that of the inside diameter of the silicone tubing. The BSA release behaviour from these scaffolds was studied in DMEM at 37 °C. The initial burst release was reduced and the release was prolonged in all microsphere-embedded scaffolds (Figure 2.7). The release curves of scaffolds follow a trend similar to alginate microspheres, but the rate of BSA release was decreased. As expected, BSA released from alginate scaffolds containing microspheres made using the incorporation protein loading method is slower than from the ones containing microspheres made with incubation methods. For microspheres prepared with each loading method, chitosan coating results in slower release after the initial burst stage. Although the time interval of initial burst release was not retarded, the percentages of total loaded BSA was reduced during that earlier period. This phenomenon could be due to the fact that alginate hydrogels surrounding the microspheres provided a barrier for fast ion diffusion. However, the dissolution and swelling rate of alginate hydrogels are expected to be rapid in medium containing monovalent cations that can substitute for calcium [133]. As a result, the fast dissolution of alginate in DMEM eventually leads to fast diffusion of ions into microspheres. On the other hand, the freeze-dried microspheres have a larger swelling rate than wet ones [134]. The quick expansion in microsphere volume could change the bulk network of alginate hydrogel, which in turn could accelerate the diffusion of BSA and other ions. The decrosslinking and dissolution process of alginate microspheres and hydrogels impose effects on each other, and thus influence the BSA release kinetics together. It can be concluded that BSA release was affected by the combined physicochemical properties of alginate microspheres and hydrogels.

Chapter 3. Alginate microspheres for release of IgG

3.1. Introduction

As described in chapter 2, the alginate microspheres prepared using the emulsion/external gelation method have large diameters and wide size distribution. Many researchers have attempted to produce small and homogenous alginate microspheres by optimizing preparation parameters in the emulsion process. It has been reported that the diameters of alginate microspheres ranged from 500 to 800 μm in one earlier study [97], and were reduced to 8 μm [66] and 5 μm [56] in other studies by improving preparation formulations. Also, the encapsulation efficiency and release kinetics of proteins were found to be significantly influenced by the molecular weights and isoelectric points of proteins [71, 135]. Release studies of different proteins have reported that the protein chemical properties and their interaction with alginate polymers are related to their release profiles. Proteins with high p*I*, such as the growth factor bFGF, have been shown to have higher affinity with alginate hydrogels [118]. The angiogenic growth factor VEGF, which has a high molecular weight and high p*I* value, was encapsulated into alginate beads by extruding alginate-VEGF solution into $CaCl_2$ solution, resulting in a high encapsulation efficiency and sustained release of VEGF [136]. A recent *in vitro* study showed that lysozyme and chymotrypsin, both with molecular weights lower than 25 kDa and high p*I* values, had lengthy and extended release from alginate beads produced by extrusion methods, as compared with release profiles of BSA [105]. However, the release behaviours of IgG, which is a high p*I* protein with large molecular weight, from alginate microspheres prepared with emulsion/external gelation methods have not been well studied. It therefore seems worthwhile to investigate microencapsulation techniques of a variety of proteins for accomplishing prolonged protein release and tailoring protein release to fit different conditions.

Local and sustained protein release has been achieved by using microsphere-embedded polymeric scaffolds *in vitro* [118, 119]. Alginate microspheres have been demonstrated as potential delivery vehicles for BSA, and we have shown in chapter 2 that alginate microsphere incorporating scaffolds extend the protein release rate. It is of interest whether preparation parameters for alginate microspheres could be further improved, and whether the typical burst

effect of alginate microspheres could be reduced effectively and proteins could be released in a controllable manner by using microsphere incorporated scaffolds to release a different protein.

The previous study shows the necessity of further investigation of parameters of alginate microsphere preparation and release kinetics of different proteins. In the present study, the encapsulation of immunoglobulin G (IgG), a protein with high molecular weight and high p*I*, in calcium alginate microspheres coated with chitosan was investigated. The IgG-loaded alginate microspheres were prepared using a smaller emulsion volume, in order to decrease the amount of protein needed in microsphere preparation. The physical properties, encapsulation efficiency, and *in vitro* release kinetics of IgG encapsulating alginate microspheres were analyzed. Also, microsphere-incorporated alginate cylindrical hydrogels were fabricated using alginate microspheres with or without chitosan coating. The alginate cylindrical hydrogels were prepared by extruding a microsphere-alginate mixture into a high concentration of $CaCl_2$, which provides the benefit of fast gelation of alginate exposed to calcium ions. The release of IgG from this alginate hydrogel and the influence of chitosan coating and hydrogel composition were examined *in vitro*.

3.2. Materials and methods

3.2.1. Materials

All materials used were obtained from the same source described in Chapter 2. Immunoglobulin G (IgG) from rabbit serum was obtained from Sigma (Oakville, Canada). DyLight 800 antibody labelling kit was purchased from Thermo Fisher Scientific Inc (Nepean, Canada).

3.2.2. Preparation of chitosan coated and uncoated alginate microspheres

Sodium alginate was dissolved in ultrapure water at room temperature to obtain solution of 5% (w/v). Alginate microspheres were prepared using the same emulsion method described in chapter 2, but at a smaller scale. Briefly, 50 μL of Span 80 was added into 1 mL of paraffin oil in one well of a 24-well plate, and mixed using a mechanical stirrer. Three hundred μL of 5% alginate solution was then added dropwise while stirring at 1300 rpm on a mechanical stirrer.

After 1 hour of constant mixing, 150 µL of 10% $CaCl_2$ was gradually dropped into the homogeneous emulsion solution, and stirred for 1 hour. Pure isopropyl alcohol (300 µL) was added to harden the alginate microspheres that were formed. The stirring was continued for 10 minutes. Microspheres were then collected by centrifuging at 1000 g for 15 minutes, and they were washed twice with isopropyl alcohol and once with ultrapure water. Alginate microspheres were freeze-dried as described in chapter 2. All batches were prepared in quintuplicate. The alginate microspheres were coated with 0.1% chitosan as described in chapter 2 except microspheres were used directly after washing without lyophilisation.

3.2.3. Physical characterization of alginate microspheres

Lyophilized alginate microspheres were observed with SEM as described in chapter 2.

3.2.4. Preparation of IgG loaded alginate microspheres

3.2.4.1. Labeling IgG with near infrared dyes

The labeling of IgG with DyLight 800 dyes was performed as described in the manufacturer's instructions. The IgG concentration was also determined according to the instructions. DyLight 800 labeled IgG was aliquoted and stored in -20 °C until use.

3.2.4.2. Encapsulating labeled IgG within alginate microspheres

Protein-loaded alginate microspheres were prepared by the incorporation method described in chapter 2. Briefly, 27 µg of labeled IgG in PBS was added to 300 µL of 5% alginate solution, and mixed by vortexing. The alginate solution containing labeled IgG was used to form a water-in-oil emulsion as described in previous section.

3.2.5. Release studies of IgG loaded alginate microspheres *in vitro*

Approximately 2.5 mg of alginate microspheres was placed into one 1.5 mL microcentrifuge tube, and suspended in 1 mL of 20 mM Tris buffered saline (TBS, pH 7.4) with 2 mM $CaCl_2$. The microsphere suspension was gently shaken horizontally at 100 rpm and maintained at 37 °C. Samples were periodically removed from the shaker, and centrifuged at 1500 *g* for 15 minutes. An aliquot of 10 µL of the supernatant was removed and transferred to, and diluted in a 96-well plate. The same amount of TBS was added to each microtube to replace

the removed supernatant, and the microspheres were resuspended for further protein release. The 96-well plate containing supernatant samples was imaged using a LI-COR Odyssey infrared imaging system (Guelph, ON) in the 800 nm channel, and the integrated fluorescence intensities for each well were quantified using the LI-COR Odyssey infrared imaging system software. The obtained integrated intensities were analyzed using Excel (Microsoft Corp., Redmond, WA) and Prism (GraphPad Software, San Diego, CA) software. Known amounts of labeled IgG were placed into separated wells of the same 96-well plate to serve as standards during each imaging. Plates were protected from light before imaging and scanned as soon as possible after they were removed from the shaker. Five samples in each condition were prepared.

3.2.6. Preparation of microsphere-integrated alginate scaffolds

Protein-loaded alginate microspheres were used directly after the final washing step to reduce the loss of fluorescence intensity of the DyLight 800 label. Known volumes of wet microspheres were dispersed into 2% (w/v) alginate solution to get a final microsphere concentration of 5% or 7.5% (v/v). The suspensions were immediately injected through Silastic tubing with an inner diameter of 1.47 mm into a glass plate containing 1 M $CaCl_2$ solution. The tubing was immersed in the $CaCl_2$ solution during the injection process. The microsphere-alginate suspensions were eluted through the tubing and gelled immediately when they contacted the $CaCl_2$ solution. The formed cylindrical shaped gels were allowed to further crosslink in 1 M $CaCl_2$ solution for 15 minutes, and were then washed in ultrapure water to remove unreacted $CaCl_2$ residue. The completely gelled scaffolds were cut to form cylinders with a length of 1 cm.

3.2.7. Release studies of IgG in microsphere-integrated alginate scaffolds *in vitro*

The cylindrical scaffolds used for release studies were approximately 1.2 mm in diameter and 1 cm in length. The scaffolds made with different alginate microspheres were placed into 24-well plates and immersed in 0.5 mL of 20 mM TBS plus 2 mM $CaCl_2$. Scaffolds made with blank alginate microspheres were used as controls. Plates were incubated at 37 °C under gentle agitation on a horizontal shaker. At each scheduled time point, 10 µL of the medium was collected and replaced with the same volume of fresh medium. The collected samples were imaged in 96-well plates using the LI-COR Odyssey infrared imaging system and analyzed as described above. All measurements were carried out using five samples from each condition.

3.2.8. Determination of encapsulation efficiency of labeled IgG

Alginate microspheres loaded with labeled IgG were suspended in 1 mL of 20 mM TBS (pH 7.4), and incubated at 37 °C for 3 days with gentle agitation to promote complete protein release. Microspheres were separated from the incubation medium by centrifugation at 1500 *g* for 15 minutes. The supernatants that were collected were imaged by a LI-COR Odyssey infrared imaging system (Guelph, ON), and the amount of labeled IgG was analyzed as described in section 3.2.5. The fluorescent intensities of alginate microspheres after 3-day incubation were also observed by light microscopy to ensure complete release of labeled IgG from microspheres. The encapsulation efficiency (EE) was calculated from equation 3.1, and three batches were used for calculations.

$$EE\% = \frac{\text{Mass of loaded IgG}}{\text{Mass of added IgG}} \times 100 \qquad \text{(Equation 3.1)}$$

3.3. Results and discussion

3.3.1. Properties of alginate microsphere

Alginate microspheres were successfully prepared using the small volume emulsion system with and without labeled IgG. The scanning electron micrographs show spherical shaped microsphere with approximately diameter of 1 to 3 μm in a relative narrow size distribution (Figure 3.1 and 3.2). The SEM images demonstrate that the emulsion process and formulation parameters that we used resulted in alginate microspheres with good morphological characteristics and small size. Alginate microspheres prepared with and without incorporation of labeled IgG show no distinguishable differences in shape and surface morphology.

The formation of small and uniform alginate microspheres using this emulsion system could be affected by several process parameters, such as polymer concentration, polymer-to-oil ratio, emulsifier concentration, stirring speed, and volume of processing medium. The surfactant concentration, which is 5% (w/w), was kept constant through the experiments. It has been found that higher emulsifier concentration in water-in-oil emulsion can reduce the diameters of the resultant microspheres [137]. Comparing the microsphere preparation formulation in section 2.2 and 3.2, the processing medium, and the stirring speed were altered. The processing medium was reduced from 50 mL to 1 mL, and the container used was changed from a beaker to a 24-well

plate, which result in smaller and more uniform microspheres. However, the volume ratio between alginate solution and paraffin oil and the concentration of emulsifier remained unchanged. The lowest effective Span concentration that can form a stable emulsion is 2%. The Span 80 concentration used, 5%, was high enough to decrease the surface tension between the aqueous droplets and surrounding oil medium, and form stable alginate droplets in paraffin oil during the emulsion process. Therefore, the polymer-to-oil ratio and emulsifier concentration are likely not the major contributors to the size and uniformity of microspheres. However, the geometry of the container, the stir bar size, and the sample volume should be taken into consideration for the changes in size and uniformity of microspheres. The reduction in the volume of processing medium and container could increase the effectiveness of mechanical stirring of stir bars, which in turn can produce smaller and more uniform alginate droplets during the emulsion process. Also, the increase of stirring speed from 1200 to 1300 rpm could also contribute to the more effective dispersion of alginate solution in the paraffin oil. A higher stirring speed can provide more energy for dispersion, which reduces the size and increases uniformity of microspheres [138]. Formation of spherical, small, and uniform aqueous droplets in the oil phase is a key step for preparing microspheres using the emulsion technique.

Isopropyl alcohol is commonly used as a dehydrating agent after emulsification process, and as washing medium in alginate microsphere preparation [120, 139]. It has been found that isopropyl alcohol hardened and washed alginate microspheres generally have smooth surfaces, spherical shape, and reduced aggregation effects.

We used the small scale microsphere preparation method and the incorporation method for protein loading to prepare IgG incorporating alginate microspheres. Table 3.1 shows that this process resulted in a high encapsulation efficiency. Due to the insolubility of protein in isopropyl alcohol, protein loss during washing cycles could be minimized, which in turn increases the protein encapsulation efficiency. The results obtained show that using isopropyl alcohol to harden and wash alginate microspheres in which encapsulated protein is insoluble is suitable.

Table 3.1. Alginate microspheres prepared and IgG encapsulation efficiency.

Condition abbreviations	IgG encapsulation	Encapsulation efficiency (%)	Coating
Alg-Un	No	N/A	No coating
Alg-Ch			0.1% chitosan
IgG-Un	Yes	89.4 ± 6.4	No coating
IgG-Ch			0.1% chitosan

The EE percentages are shown in mean ± S.D. (n = 5).

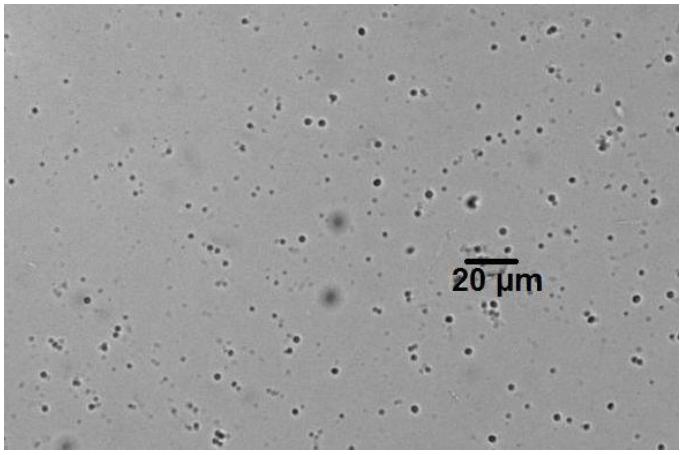

Figure 3.1. Optical photomicrograph of alginate microspheres prepared with 10% $CaCl_2$ in small volume.

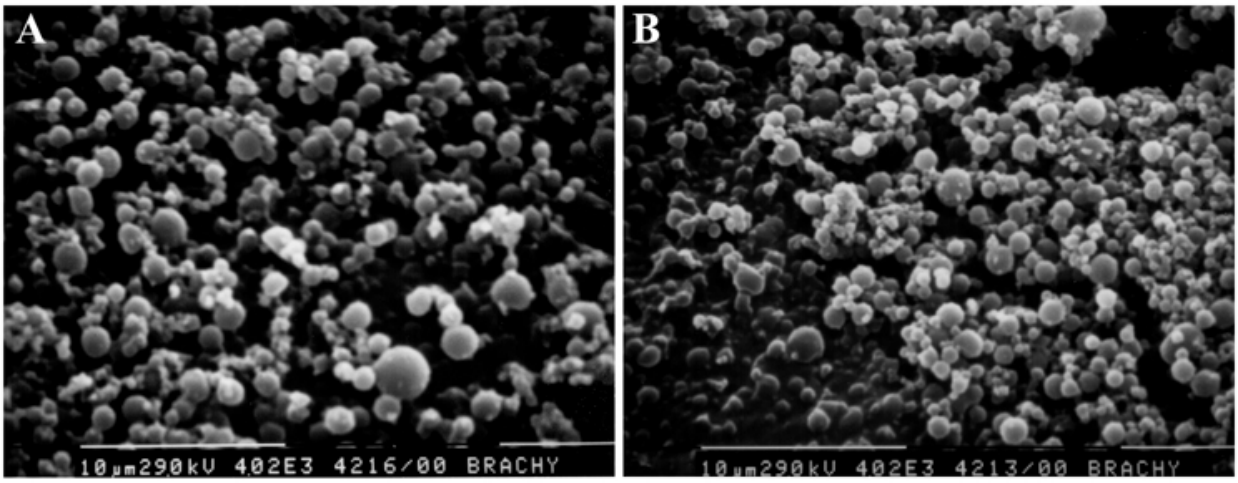

Figure 3.2. SEM images of (A) alginate microspheres and (B) IgG loaded alginate microspheres.

3.3.2. Release kinetics of IgG from alginate microspheres *in vitro*

In vitro release of labeled IgG from alginate microspheres with or without chitosan coating was investigated. The releasing medium contained 2 mM $CaCl_2$ in order to study the dissolution, disassociation, and degradation of alginate microspheres under physiological conditions. At predetermined time intervals, only 1% of total releasing medium was removed for analysis, in order to not affect the accumulated concentration of IgG protein in the releasing medium. The changes in the concentration of IgG protein in the surrounding medium produce concentration gradient between the IgG encapsulated inside microspheres and in the surrounding medium, which artificially promotes protein release into a medium with lower IgG concentration. To better illustrate the accumulated IgG release, no concentration gradient was induced in the release studies.

The accumulated IgG release was measured in TBS (pH 7.4) with the presence of 2 mM $CaCl_2$ and the release profiles are shown in Figure 3.3. The prolonged and sustained release profiles of IgG from chitosan coated and uncoated alginate microspheres show that IgG proteins were successfully encapsulated within the interior of the alginate microspheres. The release of IgG from alginate microspheres was rapid during the first 2 to 5 hours with or without chitosan coating, which is presumably caused by the fast release of IgG adsorbed onto microsphere surface, and the initial burst of alginate microspheres once they were introduced into aqueous solution with neutral pH. However, the IgG was completely released from alginate microspheres within 160 hours, where only 50% of IgG was released from chitosan coated alginate microspheres during that time. Also, a more noticeable slowed release is observed in the chitosan coated microspheres after the initial burst effect. Therefore, the chitosan coating could effectively reduce the initial burst effect, and slow down the overall releasing time.

The release and diffusion of encapsulated IgG could be influenced by many factors, such as the size of protein, the pore size of the microspheres, the dissolution of alginate molecules, the breakdown of alginate polymer, and the degradation rate of alginate. The release profile of uncoated alginate microspheres is relatively smooth, potentially indicating that swelling and dissolution of small and uniform microspheres prepared with high alginate concentration are slow in TBS with added calcium ions. The release curves done in a different buffer solution, PBS, showed a greater swelling and a more obvious burst effect comparing to release curves done in

TBS. This phenomenon could be caused by the increased extraction (and precipitation) of calcium ions in microspheres by phosphate ions in PBS. On the contrary, Tris is an organic compound which does not form insoluble precipitates with calcium ions.

The release of IgG was slowed down after the chitosan coating process, which indicates that the chitosan coating was successful and the chitosan membrane outside the microspheres protects them from the surrounding aqueous medium. The chitosan membrane is formed due to the electrostatic interaction between the carboxyl groups of alginate and the amine groups of chitosan. The chitosan coating process was done under an acidic environment (pH 5) where the amine groups of chitosan are positively charged and carboxyl groups of alginate are negatively charged. When the chitosan coated alginate microspheres were suspended in buffer at pH 7.4, the chitosan molecules are deprotonated, and thus, the charge density of chitosan molecules was reduced. Therefore, the electrostatic interaction between amine groups and carboxyl groups was weakened, which leads to dissolution of chitosan molecules from the microsphere surface. However, the release profiles reveal that the disassociation of microspheres and their chitosan membrane is slow enough so that IgG release is still retarded. Also, the prolonged IgG release from chitosan coated microspheres could be caused by a diminished swelling effect of microsphere core, which is the major cause of the initial burst effect.

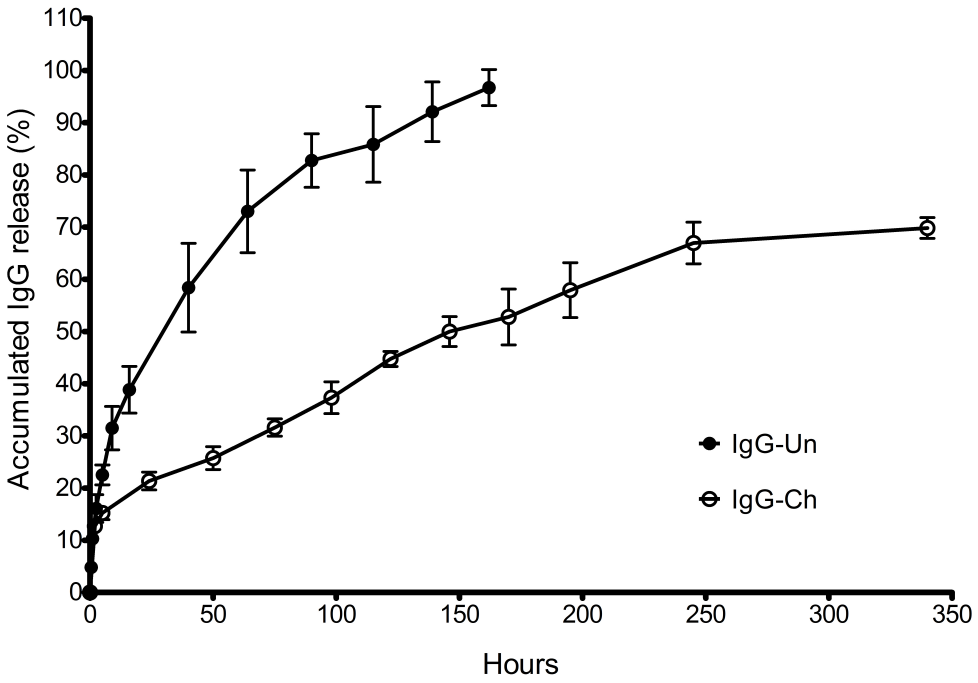

Figure 3.3. Percent cumulative release of IgG from alginate microspheres (•), and chitosan coated alginate microspheres (○) in TBS at pH7.4 and 37 °C. Values represent means ± S.D. (n = 5).

3.3.3. Release kinetics of IgG from microsphere-containing alginate scaffolds *in vitro*

Alginate solutions containing suspended microspheres extruded into 1 M $CaCl_2$ solution through silicone tubing solidified instantly and formed opalescent gels upon contact with the $CaCl_2$ solution. While these gels were further crosslinked in $CaCl_2$ solution, shrinkage of gels was observed. It has been found that penetration of calcium ions into the interior of alginate hydrogels induces water diffusion toward the outside of the gels [140]. The crosslinking of the exterior of alginate gels is extremely fast, but the gelation of the interior is highly dependent on the diffusion rate of calcium ions, and therefore the size or the thickness of hydrogels [141]. The rapidly crosslinked outer layer of alginate gels essentially forms a membrane between exterior calcium ions and interior carboxyl group of alginate molecules, thus creating a barrier for further diffusion of calcium ions into the interior of gels. The limitation of calcium ions diffusion slows down the crosslinking reactions inside the hydrogels, meaning a complete crosslinking process could take up to one day depending on the size of gels [140]. However, a significant amount of encapsulated protein in alginate microspheres diffuse out when they are washed with 50 mM $CaCl_2$ [93]; thus a solution with high concentration of calcium ions does not prevent the leakage of protein. As a result, exchange of calcium ions and water molecules during long term crosslinking could induce diffusion of encapsulated protein out of alginate microspheres, which could leak out of alginate gels. In fact, it has been proven that a 10 to 20 minute gelation time could produce a small stable alginate hydrogel [94, 141]. Therefore, the total gelation time in $CaCl_2$ solution used was 20 minutes. It was assumed that a high concentration of $CaCl_2$ could accelerate the crosslinking process, although there could be a threshold to the $CaCl_2$ concentration required. The $CaCl_2$ concentration chosen, 1 M, could be the main cause for the contraction of the alginate cylinders due to the osmotic effect. Indeed, the diameters of alginate cylinders obtained were approximate 75-80% of the diameter of silicone tubing, which further shows that short term gelation can be effective. The results show that alginate cylinder scaffolds maintained their cylindrical shape over the 2-week study. Although they appeared to be more translucent and more fragile over time, possibly due to swelling of hydrogels and breakage of microspheres, alginate hydrogels prepared are quite stable at the given experimental conditions.

Alginate microspheres encapsulating IgG were incorporated in a cylinder-shaped alginate scaffold at different densities. Alginate gel scaffold mixed with 5% or 7.5% of either non-coated

and chitosan coated alginate microspheres were evaluated for their protein release kinetics (Figure 3.4). For all of the incorporated scaffolds prepared using 5% alginate microspheres (IgG-Un-5%), 7.5% alginate microspheres (IgG-Un-7.5%), 5% chitosan coated alginate microspheres (IgG-Ch-5%), and 7.5% chitosan coated alginate microspheres (IgG-Ch-7.5%), the rates of protein release were decreased comparing to IgG released form microspheres alone. The results are reported as percentages of IgG released compared to the total IgG encapsulated in microspheres for each centimeter of scaffolds. By the last day of release study, cumulative release of IgG from scaffolds containing IgG-Un-7.5% microspheres was 34.10±0.83% of loaded IgG, from scaffolds containing IgG-Un-5% was 27.08±0.68% of IgG, from scaffolds containing IgG-Ch-7.5% was 18.68±0.52% of IgG, and from scaffolds containing IgG-Ch-5% was 14.03±0.68% of IgG. The release profiles of all microsphere-incorporated gel scaffolds reveal that IgG release was retarded and elongated compared to microspheres alone, and the initial burst effects of microspheres were diminished possibly due to the protection from alginate hydrogel.

The amount of microspheres embedded in alginate gel scaffolds affects the IgG release kinetics. As expected, more IgG is released from scaffolds embedded with more IgG loaded microspheres. Comparing cumulative IgG release between scaffolds with 5% and 7.5% of microspheres, approximately 1.5 times more IgG released from scaffolds with 7.5% microspheres than with 5% microspheres. This result also suggests that differential degradation and dissolution of alginate hydrogels with changing microsphere density did not impact the protein release kinetics significantly over the course of the release study. It is possibly due to the fact that calcium crosslinked alginate hydrogels are stable in neutral buffer with added calcium ions. Slower release of IgG is observed in scaffolds made with chitosan coated microspheres than uncoated microspheres. In all cases, IgG was released over extended time period from embedded hydrogels comparing to microspheres.

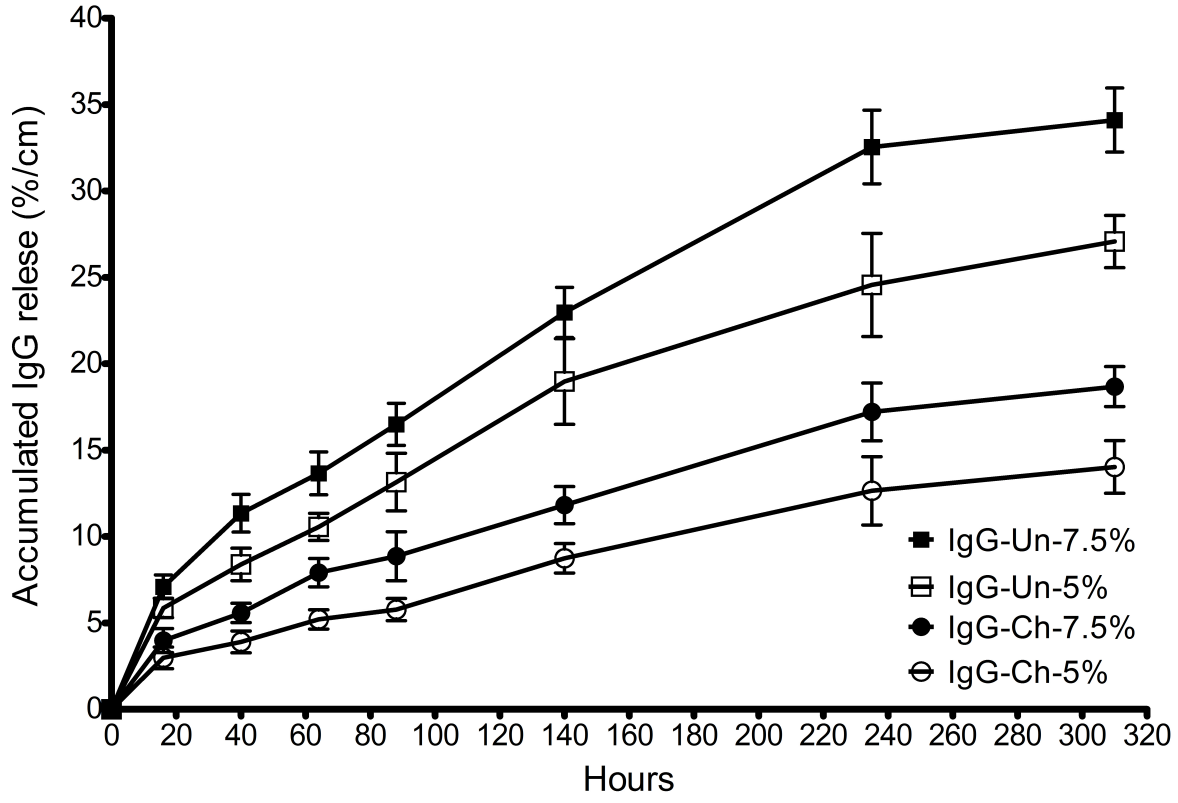

Figure 3.4. Percent cumulative release of IgG from one centimeter microspheres-incorporated scaffolds in TBS at pH7.4 and 37 °C. Alginate scaffolds prepared with different IgG-loaded microspheres: 5% alginate microspheres (□), 7.5% alginate microspheres (■), 5% chitosan coated alginate microspheres (○), and 7.5% chitosan coated alginate microspheres (●).Values represent means ± S.D. (n = 5).

Chapter 4. General discussion

4.1. Preparation of alginate microspheres

Alginate microspheres can be prepared by a two-step process: emulsification and external ionic gelation [66, 120, 121, 142]. In a general microsphere preparation process, the alginate solution was added slowly into an oil phase containing proper surfactants and stirred to form emulsion droplets, which is followed by gelation of formed droplets induced by addition of $CaCl_2$ solution. The solidified alginate microspheres are then hardened and washed in dehydration agents. It has been found that formation of alginate microspheres is affected by the formulation parameters used in the emulsification process. Various formulation and preparation parameters, such as surfactant properties [143], stirring speeds [93], stirring time, type of oil [144], polymer concentration [66, 121], and polymer-to-oil volume ratio, have been reported to play an important role in the formation of an emulsion. Also, the size and shape of alginate microspheres formed are affected by the volume and concentration of calcium chloride used in the ionic gelation step [121]. Generally, all alginate microspheres obtained from the current study have a spherical shape and smooth surface. However, alginate microspheres prepared with different formulation parameters showed variations in morphology and protein release kinetics. The size and size distribution of the microspheres vary with the changes in the emulsification/gelation formulation, and protein-loaded microspheres exhibit different release profiles due to their distinct physicochemical properties.

4.2. Effect of emulsifier concentration

The use of surfactants is critical for dispersion of two immiscible liquids to a stable emulsion. Span 80, an oil soluble surfactant, has been used to produce stable aqueous droplets in paraffin oil due to its ability to reduce the high interfacial tension when its concentration is equal to or above its critical micelle concentration (CMC) [52, 54]. Although aqueous phase could be dispersed as small globules in the oil phase with low Span 80 concentration, the stability of these globules is low causing distortion and coalescence [145]. Hence, the surfactant concentration that is used is generally equal or greater to its CMC.

The CMC of Span 80 has been established to be 0.5% (w/w) at a water-paraffin oil interface, and a concentration greater than the CMC can effectively produce water-in-oil

microemulsions [146]. It also has been reported that high concentration of Span 80 could produce highly stable emulsions in paraffin oil at short stirring time and hinder the coalescence of droplets, which is a crucial factor for formation of spherical and uniform aqueous droplets. In fact, sorbitan monoleate or Span 80 was found to produce smaller microspheres, since its straight chain fatty acid can effectively adsorb onto the alginate globules and pack them closely [147].

In addition, the properties of Span 80, for example, hydrophile-lipophile balance (HLB) has been found to influence the size and shape of microspheres obtained using emulsification methods [143, 145]. The influences of different of surfactants have been investigated, and the results demonstrated that non-ionic surfactants with lower HLB, such as Span 80, have a higher ability to produce smaller alginate microspheres [66, 143]. Previous studies also have shown that emulsifiers with one single long straight chain fatty acid and lower HLB produce smaller macromolecule-loaded microspheres with higher encapsulation efficiencies and slower release rates [120, 143, 145].

The paraffin oil/Span 80 emulsion system was first introduced for preparing covalently crosslinked chitosan microspheres [116]. Chitosan microspheres with diameters ranging from 274 to 450 μm were prepared in 100 mL of paraffin oil containing 0.5% Span 80 stirring at 700 rpm. When the emulsion mixture of chitosan solution and paraffin oil containing 1% Span 80 was stirred at 1000-3000 rpm, the diameters of chitosan microspheres were reduced to about 10-14 μm [117]. A recent study used dispersion of 150 mL of chitosan solution-paraffin oil with 2% Span 80 to obtain microspheres with mean diameters of 20-31 μm depending on the crosslinker concentrations [148]. Alginate microspheres were also prepared with paraffin oil/Span 80 emulsion system [93]. These alginate microspheres produced in paraffin oil with 1% Span 80 stirred at 400 rpm, but gelled internally, have diameters of 26.0±9.7 μm.

An emulsion mixture with a high concentration of Span 80 in paraffin oil was tested for its potential to produce externally gelled alginate microspheres. For this reason, 5% of Span 80 was chosen as the emulsifier in order to sufficiently reduce the interfacial tension between alginate phase and paraffin oil phase. Small and stable emulsion globules were produced. As expected, the majority of alginate microspheres we prepared using the methods described in chapter 2 are smaller than 30 μm (Figure 2.2 and 2.3), and smaller than 3 μm in chapter 3 (Figure

3.2). Only small aggregations of microspheres are observed in optical micrographs (Figure 2.1 and 3.1).

Alginate microspheres prepared in emulsion system using Span 85, Tween 85, Sodium desoxycholate, PVA, or Pluronic F68 in iso-octane have been reported to have diameters ranging from 1 to 150 μm, and were observed to have clumps of smaller microspheres [66, 120]. Nevertheless, one study stated that an emulsion system made with low concentrations of combined Tween 80 and sodium lauryl sulphate in vegetable oil could produce alginate microspheres with a mean diameter of 18 μm [149]. The use of a mixture of Tween 80 and sodium lauryl sulphate was an attempt to prevent spheres from coalescing during the emulsion process, and to reduce the gelation time after the addition of $CaCl_2$ solution. Other studies have proven that increasing the Span 80 concentrations could efficiently reduce the probability of aqueous globule coalescence [150], and the subsequent diffusion of calcium ions into alginate droplets is relatively rapid due to their small size.

The results obtained in this study confirm that high concentration of Span 80 can produce small spherical alginate microspheres with no aggregations. In this present study, 5% of Span 80 was used in all emulsification process; however, the diameters of alginate microspheres are larger than 20 μm when the $CaCl_2$ concentration was lower (Table 2.2), but smaller than 3 μm when the $CaCl_2$ concentration was raised and stirring speed was increased (Figure 3.2). Therefore, increasing the surfactant concentration well beyond its CMC could facilitate reducing the size of emulsion droplets if other influential factors of emulsion formation remain unchanged. Also, the size and shape of micelles not only rely on the surfactant concentration, but also on the surfactant molecular architecture and the physical interaction between the surfactant and the two phases. The experimental results of this research revealed that the Span 80 concentration used maintains the spheroidal shape of the microspheres, but displays limited effects on microsphere size and size distribution.

4.3. Calcium chloride concentration

After the micelles have been formed in an emulsion mixture, ionic crosslinking of alginate using internal or external calcium sources is required to stabilize the spheroidal shape and to form solid microspheres. The method of using calcium carbonate as an internal gelation

source requires activation of the crosslinking process by glacial acetic acid, which causes a sudden pH reduction to 5 in the emulsion mixture [93, 151]. Stability or resistance of encapsulated materials to the quick change of pH are needed for using this internal gelation method in the emulsification process.

Internal gelation methods produce homogeneous but more porous alginate gels, since the calcium ions are uniformly distributed in alginate solution initially, and then the proton-induced gelation occurs at the gel surface moving into the inner core [151]. Using calcium ions as the external source is an alternative way for alginate crosslinking because it creates a mild condition for ionic interaction at neutral pH. External gelation methods utilize the self-assemble abilities of two phases between surfactants; thus when the aqueous $CaCl_2$ solution is introduced into emulsion system, the system is disrupted to allow the distribution of calcium ions into aqueous alginate micelles already formed and then return to the system to a new equilibrium while stirring. In the external gelation methods, the initial contact points of alginate molecules and calcium ions are at the sphere surface and the gelation process depends on the movement of free calcium ions and alginate molecules. As a consequence, a less permeable and smooth surface is formed initially, which immobilizes alginate molecules and encapsulants near the gel surface but reduces diffusion of calcium ions into the sphere core. However, external gelation methods produce spheres with more compact and smooth surfaces than internal gelation methods, which provide alginate spheres a higher resistance to ion diffusion [151, 152].

The low permeability of alginate microspheres is related to the release rate of encapsulants; limiting the permeability of crosslinked alginate microspheres is assumed to reduce the diffusion rate of encapsulated protein. In fact, the BSA release profiles of alginate microspheres prepared with 1% and 10% of $CaCl_2$ solution demonstrate that the release of protein depends on the amount of calcium ions used for crosslinking (Figure 2.6). In all cases less BSA was released or diffused into medium during the initial stage when the concentration of $CaCl_2$ was increased.

Since the gelation or calcium ions uptake of an alginate gel was found to reach a plateau after 30 minutes using different calcium concentrations in external gelation [94, 153, 154], the gelation time used in this study is assumed to create microspheres at equilibrium where no calcium ion exchange occurs at the end of gelation time. The differences in the BSA release

profiles of alginate microspheres are presumably caused by the amount of calcium ions present during the gelation process. Higher concentration of calcium cations produces alginate microspheres with a highly crosslinked exterior, which in turn decreases the permeability of microspheres to further calcium entry. However, Figure 2.6 also indicates that the concentration of $CaCl_2$ used for gelation has more effects on the BSA release at the earlier stage of release profiles. This suggests that the level of permeability of microspheres is corresponding to the diffusion rate of encapsulants at initial release stage.

This observation could be explained by the fact that the chemical properties of alginate can affect the physical properties of hydrogels formed from it [87]. The alginate used for this study has been reported to have low viscosity, low molecular weight (~50 kDa), and an M/G ratio of 1.67 [129]. Previous studies have found that hydrogels made with high M/G ratio alginate showed a higher swelling rate and higher solute leakage than low M/G ratio when stored at neutral pH [151, 155, 156]. Also, low molecular weight or low viscosity alginate with low G content produce unstable and weak hydrogels. Since the calcium ions only form complexes with G blocks of alginate, alginate with low G content forms less ionic bonding with calcium [73], which creates more flexible and elastic hydrogels for ion flux but increases the swelling rate when equilibrated with neutral buffer [157].

The present study reveals that all microspheres prepared with high M/G ratio alginate have high BSA release rates during the time course of the release study (Figure 2.6), which suggests high swelling properties of microspheres when stored in PBS at pH 7.4. Alginate microspheres prepared with 10% $CaCl_2$ showed slower BSA release and a reduced burst effect during the earlier stage, possibly due to the increased permeability of microspheres. However, the releases of BSA rose to higher levels and reached a plateau more quickly despite the amount of $CaCl_2$ used during the later stages of release. The results indicate that increasing the crosslinking degree of alginate microspheres could diminish initial burst effects and delay the protein release for a short period of time but could not reduce water diffusion in the long run, if alginate with low viscosity and low G contents is used.

The effect of $CaCl_2$ concentration used in the alginate droplet solidification process on the size of microspheres has been studied. Earlier studies have showed that there are shrinkage effects induced by calcium ions on bulk alginate hydrogels upon ionic crosslinking [87, 151,

152]. Syneresis of alginate hydrogels has been found to be greater when the concentration of calcium ions is higher. More available calcium cations permit a higher degree of ionic interactions between alginate molecules and cations, which induces conformational changes of the alginate polymer. This may be the cause of contraction and dehydration of alginate hydrogels given enough time for calcium ions uptake by hydrogels.

A similar syneresis effect was observed in this study when the concentration of $CaCl_2$ used for gelation was increased. The diameters of alginate microspheres solidified with 10% $CaCl_2$ are significantly smaller than 1% $CaCl_2$ (Table 2.2). Figure 2.1 shows that there is a higher degree of shrinkage with all alginate microspheres prepared with 10% $CaCl_2$ than 1% $CaCl_2$, indicating the size of microspheres can be reduced by strengthening the syneresis of alginate polymers. Also, the size or size distribution of alginate microspheres could be controlled by the amount of calcium ions used for gelation process, although the syneresis process cannot be precisely regulated. In general, higher concentration of $CaCl_2$, when used as external calcium source, is expected to increase the syneresis of alginate hydrogels at the expense of losing the internal polymer structures of the alginate.

4.4. Effect of stirring speed

Another aspect of the emulsification process affecting the size and shape of microspheres is the shear force. In the current study, shear force is determined by the stirring speed. Past studies aiming at investigating different emulsion parameters have shown that increasing the stirring speed could reduce the mean diameters of the microspheres that are produced [58, 158, 159]. A high stirring speed can provide more dispersive force and turbulent stress, which tend to break up the dispersed emulsion droplets [160]. Figure 2.3 and 3.2 show alginate microspheres prepared with 10% $CaCl_2$ but using different emulsion process. The size range of alginate microspheres in Figure 2.3 are estimated to be 1 – 13 μm, while in Figure 3.2 are 0.5 – 3 μm. The alginate to oil volume ratio used is 1:4 in Chapter 2, and 1:3.3 in Chapter 3. It was found that the volume ratio between dispersed phase and continuous phase does not affect the diameter of emulsion droplets significantly for long term mixing [161]. Also, these ratios only have small variations between the two emulsion processes, thus it is not considered as the major cause of the reduced microsphere size. The decrease in microsphere diameters and narrow size distributions are mostly caused by the increase in dispersion force during the emulsification process.

Since both emulsification processes were done by mechanical stirring using stir bars, the overall volume of the emulsion mixture is important for effective mixing. Previous study has demonstrated that emulsion droplet size is independent on the emulsion volume if mixing speed is extremely high, such as 15,000 rpm [159]. Nevertheless, the stirring mechanism used in this study was the use of magnetic stir bars rotating at a much lower speed; thus, the total emulsion volume became a factor for the size of emulsion droplets. A large mixing volume could create more resistant force against stir bars, which causes reduction in dispersive force throughout the mixture. Also, it is highly likely that the stir bars cannot produce enough turbulent stress to create evenly distributed force throughout the emulsion mixture, which leads to the formation of unevenly sized emulsion droplets. When the emulsion volume was scaled down from 50 mL to 1 mL and the stirring speed increased from 1200 rpm to 1300 rpm, smaller and monodispersed microspheres were obtained. This suggests that the movement of stir bars was less restricted by the viscous emulsion mixture and turbulent stress was relatively uniformly distributed throughout the mixture, which all contribute to the formation of small and uniform alginate microspheres. In fact, the fragmentation of emulsion droplets occurs when the shear force is larger than cohesive force of the droplets, and the size of microspheres is directly related to the size of emulsion droplets [162, 163]. These results indicate that the size and size distribution of microspheres formed using emulsion techniques are strongly influenced by the magnitude and distribution of dispersive forces throughout the emulsion mixture. Past experimental results also show that microspheres prepared with high stirring speed had smaller burst effects and sustained release [164].

4.5. Loading methods

Two methods were used to load BSA into alginate microspheres: incorporation and incubation methods. In the incorporation methods, BSA was added to the alginate solution prior to the emulsification and gelation process, and the incubation methods involve immersion of pre-synthesized alginate microspheres into a BSA solution with a low concentration of sodium. Table 2.3 shows the encapsulation efficiency of BSA, and Figure 2.5 compares the effects of two protein loading methods on release profiles for alginate microspheres prepared with 1% and 10% $CaCl_2$.

Despite of amount of $CaCl_2$ used for gelation, the encapsulation efficiency for BSA is significantly lower and the release of BSA is more rapid for incubation methods than for incorporation methods. The incubation methods permit uptake of proteins by alginate microspheres due to protein concentration gradients and surface adsorption, while allowing partial decrosslinking of alginate microspheres using sodium ions. Using the incubation methods, alginate microspheres prepared with 10% $CaCl_2$ showed lower loading and encapsulation efficiency of BSA than 1% $CaCl_2$. This result could be explained by the lower permeability of highly crosslinked microspheres, which limits the amount of BSA that can diffuse into gelled microspheres.

For all microspheres loaded using incubation methods, the release profiles show higher release at the beginning stage, which may be caused by dissolution of BSA adsorbed onto microspheres or weakening of microsphere interior structures during incubation. Although the incubation methods eliminate possible BSA loss during the preparation process, the BSA loaded into or adsorbed onto microspheres during immersion is lower than expected. In fact, the incubation methods are more suitable for loading proteins with an isoelectric point equal to or higher than neutral pH [105]. Wells *et al*. [105] reported that immersing alginate microspheres in a protein solution with 0.15% NaCl could efficiently entrap high p*I* proteins due to a higher degree of ionic interaction between negative alginate and positive proteins. In the research of Wells, alginate beads produced by extruding alginate solution into $CaCl_2$ solutions were used to test the encapsulation efficiencies of two protein loading methods. However, the alginate microspheres produced by the emulsion technique in this study showed similar results regarding BSA encapsulation efficiency using the two loading methods. Since BSA is an electronegative protein at pH 7, the ability of alginate microspheres to attract BSA is dramatically reduced, and the ability of BSA to diffuse into the microsphere cores is limited.

On the other hand, the incorporation method of protein loading relies on protein interaction with alginate while mixing, and retention of protein by alginate polymers in the emulsification and washing process. The unsatisfactory encapsulation efficiencies of BSA prepared using incorporation methods (Table 2.3) could be caused by unevenly dissolved BSA powder in the alginate solution and by large BSA loss during the emulsification and washing process. However, the IgG encapsulation efficiency reached 89.4 % (Table 3.1) compared to 7.8%

for BSA both using incorporation loading methods. Apart from the improvement in microsphere preparation process, the well dissolved IgG-alginate solution and the properties of the proteins themselves could be critical causes. The Dylight 800-conjugated IgG was prepared in solution form and was evenly dispersed into 5% alginate solution before the beginning of the emulsification process. Also, the ionic interaction between electronegative alginate and electropositive IgG increases the possibility of IgG retention in alginate matrices, and thus decreases the loss of IgG during microsphere preparation. In general, incorporation loading methods provide higher encapsulation efficiencies if protein loss during preparation can be minimized, and the physical and chemical properties of proteins need to be considered if incubation methods are used.

4.6. Release properties of alginate microspheres in different release media

The release of proteins mostly depends on the diffusion rate of protein through alginate matrices and dissolution of alginate microspheres. The protein diffusion rate is mainly related to the properties of proteins themselves, and the dissolution of alginate microspheres is caused by the bulk erosion and decrosslinking. The diffusion of water molecules into alginate gels is faster than the surface degradation of alginate polymer, which leads to swelling of hydrogels. As mentioned before, the swelling is largely dependent on the molecular weight, viscosity, and G contents of alginate, where the decrosslinking process is not only related to alginate chemical properties, but also the crosslinking degree and ions surrounding the hydrogels. Moreover, the decrosslinking process accelerates hydrogel gel swelling. The replacement of calcium ions in the alginate matrices with monovalent ions and the sequestration of calcium ions by anions lead to decrosslinking of alginate microspheres. Therefore, the ionic strength and composition of the medium influence the rate of decrosslinking and degree of swelling, requiring careful consideration of the choice of release media.

At the earlier stage of this study, 10 mM PBS at pH 7.4 was used as the release medium, and 20 mM TBS at pH 7.4 was used for later studies, since the release of IgG from alginate microspheres prepared by emulsion/external gelation technique in TBS plus extra calcium ions has not been characterized. Although the emulsification conditions used to fabricate alginate microspheres and the encapsulated proteins are different in the earlier and later stage of these studies, these release profiles are still comparable in terms of overall release time. The protein

release studies done in TBS with 2 mM $CaCl_2$ showed more sustained release than in PBS. A complete BSA release was observed within 5 to 6 hours in PBS despite the amount of $CaCl_2$ used for gelation, where IgG release lasted 160 hours in TBS for non-coated alginate microspheres.

Previous experiments have revealed that alginate matrices swelled extensively if the release medium contained sodium ions, since calcium ions were replaced by sodium ions quickly and soluble sodium alginate was formed [133]. Since both PBS and TBS have sodium ions, the decrosslinking of alginate microspheres due to sodium and calcium ion exchange is not expected to be significantly different between the two media. The fast protein release and extensive swelling effect in PBS are more likely due to the sequestration of calcium ions in microspheres by the phosphate groups in PBS, which leads to formation of insoluble calcium phosphate. The high affinity between calcium ions and phosphate groups accelerates the decrosslinking process of alginate microspheres, which in turn leads to rapid swelling of microspheres and shortened release time.

However, the IgG release in TBS plus 2 mM $CaCl_2$ was tremendously extended and the initial burst of microspheres was reduced. This phenomenon could be explained by the lack of a sequestration agent with high calcium affinity and the addition of calcium ions. The purpose of adding 2 mM $CaCl_2$ is to mimic the physiological calcium concentration in DMEM tissue culture medium, which normally contains 1.8 mM $CaCl_2$ [165, 166]. The differences between the swelling properties of alginate hydrogels in PBS and Tris-HCl at pH 7.4 were studied, and our results demonstrated that swelling in PBS was mainly due to sodium and calcium ion exchange and formation of calcium phosphate, where swelling in Tris-HCl was caused by hydrolysis of hydrogels [167]. The current study showed the dependence of protein release profiles on the release media with the presence of sodium ions. The phosphate ions in PBS are likely to form precipitates with calcium ions. On the contrary, organic TBS has lower affinity to crosslinking calcium ions, which leads to a slower decrease in crosslinking density of microspheres despite the occurrence of sodium and calcium ion exchange. Thus, the composition of buffer plays an important role in protein release kinetics.

The release of protein from alginate cylinders containing embedded microspheres was examined in DMEM and TBS plus 2 mM $CaCl_2$ in Chapter 2 and 3, respectively. It has been

stated that PBS is commonly used as a release medium, but the release profiles of proteins in other media are also worth to study, since no one medium can exactly reproduce *in vivo* conditions. The intention of using DMEM as the release medium was to more closely mimic the ion profile of physiological conditions. BSA release was more rapid in DMEM than was IgG in TBS, possibly due to the complex ingredients in DMEM. The DMEM solution for cell culture consists of amino acids, vitamins, and inorganic salts [165], which all could form complexes with calcium ions. The crosslinking calcium ions in microspheres are expected to undergo ion exchange with monovalent ions or form precipitates with these inorganic salts. Although the results obtained from this study are inconclusive to determine the effect of DMEM has on alginate hydrogel dissolution and decrosslinking, protein release is expected to be faster in DMEM than in TBS plus 2 mM $CaCl_2$.

4.7. Properties of proteins

Although the two types of microsphere were prepared under different conditions, comparing the release profiles of BSA- and IgG-loaded microspheres, they illustrate that BSA-loaded microspheres have more rapid release and shorter overall release time. The large differences in the release kinetics may be explained partly by the distinctions in protein types. The diffusion rate of protein through the alginate microspheres and matrices is determined in part by the properties of proteins themselves, such as molecular weights, sizes of three-dimensional structures, isoelectric points, and interactions between the proteins and the alginate polymers [131, 135].

The average molecular weights of BSA and IgG are 67 and 155 kDa, respectively. Experiments studying the release of proteins ranging from 17.8 to 156 kDa reported that the release from alginate microspheres decreases as the protein molecular weights increases [168]. Large proteins, such as IgG, have slower diffusion rate than proteins small in molecular weights, since the permeability of alginate microspheres become a more critical factor for high molecular weight-protein diffusion.

Also, it has been found that size of 3-D structure of proteins (for example the stokes radius) has effects on the diffusion coefficients in passing through permeable gel membranes [131]. Other studies suggest that the 3-D structure of BSA is ellipsoid and that it has a high

deformation capacity, which increase its diffusion rate through chitosan coated alginate beads [135]. By contrast, IgG has a more extended three-dimensional shape, possible leading to more retarded diffusion through a hydrogel.

Previous results were obtained from alginate hydrogels or spheres produced by different methods, although IgG release from small and uniform alginate microspheres produced by the emulsion technique has not previously been reported. The previous findings are consistent with the results of this study; IgG diffuses more slowly than BSA due to the high hydrodynamic volumes of IgG, whether from chitosan coated or uncoated alginate microspheres. Another property of proteins can also be used to explain the differences in diffusion rates. The net charges of proteins are different at a given pH due to different p*I* values of each protein. BSA has a p*I* of 4.9, and IgG has a p*I* of 7.1 [169], which results in a more electronegative charge on BSA in a solution buffered at pH 7.4. The burst release profiles of electronegative BSA have been observed in alginate microspheres crosslinked with $CaCl_2$ and alginate composite microspheres [139, 170]. The negative charges on BSA impose a repulsive force on negatively charged carboxyl groups of alginate at physiological pH; as a result, alginate microspheres retain BSA for a shorter time. On the other hand, IgG presents more accessible positive charges, which leads to more ionic interactions with alginate polymer inside the microspheres. Therefore, the release profile of IgG was lengthened and the initial burst release from microspheres was reduced due to the larger molecular weight and size, and positive net charges of IgG.

4.8. Protein detection methods

Two protein detection methods were used in this study. The BSA release was detected using the Bradford assay in Chapter 2. According to the manufacturer's manual, the Bradford reagent has a lower detection limit of 1 μg/mL protein. The BSA released during the later stage of the release studies could be undetectable due to the fact that the amount of BSA protein used in loading microspheres was small and BSA-alginate microspheres have low encapsulation efficiency. After the burst period in PBS, the BSA released in the medium was approaching the lower detection limit of the Bradford assay. The Bradford assay is dependent on the protein tertiary structures and is easily affected by the detergent concentration in the assay [171]. Since the possible denaturation of BSA during the preparation of microspheres and the possible residual presence of Span 80 in the freeze-dried microspheres were not analyzed, the suitability

of using Bradford assay to detect BSA release in this experiment cannot be determined although it has been commonly used for protein detection in studying microsphere release [172-174].

The DyLight 800-IgG was detected by a LI-COR infrared imaging system in Chapter 3. The Odyssey infrared imaging system can detect near-IR dye-conjugated proteins in amounts as low as 1 nanogram [175], which provides a wide range of protein detection and reliable quantification. However, the effect of emulsification and the washing process on the stability of the Dylight 800 dye was not examined; rather the dye conjugated to IgG was assumed to be stable throughout the experiments. The effects of buffer and temperature on the fluorescent of dyes were taken into account by making standards of Dylight 800-conjugated IgG in the release medium and keeping these standards under the same condition as the IgG-loaded alginate microspheres. The experimental results show that the Dylight 800-conjugated IgG could be detected at least as low as 50 ng/mL in TBS at pH 7.4.

The methods for sampling protein release media were different for BSA- and IgG-loaded microspheres. When the BSA release media were analyzed, all of the supernatants were taken for Bradford assay after centrifugations, and were replaced with fresh buffer. The method of replacing all release medium maintains a large concentration gradient of BSA between the inside and the outside of the alginate microspheres, which promotes BSA diffusion toward the outside of microspheres. Therefore, the rapid BSA release was partially driven by the maintained high BSA concentration gradients created by replacing the release medium. In the case of IgG release, only 1% of the release medium was taken for fluorescent detection, which was considered to cause insignificant changes in the accumulated IgG concentration in the release media, and thereby more closely model likely release conditions in practice.

4.9. Effect of chitosan coating

Protein-loaded alginate microspheres were coated in 0.1% chitosan solution to lengthen the time span of protein release. In Chapter 2, complete BSA release was extended from 6 hours for uncoated microsphere to 55 hours for chitosan coated microspheres. For the IgG-loaded microspheres, IgG was fully released from naked microspheres at 160 hours, while approximately 50% of IgG was released from coated microspheres at the same time. Coating

alginate microspheres with chitosan membranes has been previously demonstrated to reduce or control protein release [93, 112].

Chitosan dissolved in diluted acetic acid is a polycationic polysaccharide, which complexes with polyanionic alginate. The pH of chitosan solution was adjusted to 5, at which the alginate is net negatively charged and the chitosan is positively charged [111]. This adjustment in pH ensures a strong electrostatic interaction between chitosan and alginate, and consequently a stable chitosan membrane can be formed around alginate microspheres. The release profiles of BSA and IgG from chitosan coated alginate microspheres demonstrate that the chitosan coating can reinforce alginate microspheres and effectively retard protein release.

4.10. Controlled release

The experimental results show that the release of protein from naked protein-loaded alginate microspheres is rapid and exhibits an unavoidable initial burst. These release profiles suggest that the protein release is largely dependent on the dissolution of alginate, ion diffusion rate, and properties of microspheres. Using $CaCl_2$ as crosslinker has the benefits of low toxicity, fast gelation time, and easy diffusion of calcium ions into microspheres. However, the disassociation of ionic bonding is fast and can be easily broken by ion exchange. The nature of ionic interaction between calcium and alginate leads to fast swelling and rupture of initially stable microspheres in neutral buffers. It has been demonstrated that the swelling and dissolution rate of alginate microspheres can be reduced by increasing the crosslinking degree. Development of advanced preparation methods can result in fabrication of uniform and small alginate microspheres, which may improve the protein release. Additionally, the chitosan coating generally enhances the microspheres and reduces the protein release, which achieves sustained release. The chemical properties of proteins can also be a critical factor for realizing sustained release.

4.11. Microspheres incorporated within alginate scaffolds

The release behaviours of BSA and IgG from microspheres incorporated within alginate cylinders are shown in Figure 2.7 and 3.4, respectively. The release profiles of BSA have an obvious initial burst release, and then undergo a slower release until complete release was reached at approximate 50 hours. As expected, the BSA release from microspheres prepared

using incubation methods was more rapid than when using incorporation methods (Figure 2.7). After the initial 7-hour-burst release, 60-70% of BSA was released from scaffolds with microspheres loaded using incubation methods, whereas 35-45% of BSA was released from microspheres loaded using incorporation methods. This observation corresponds to the release kinetics of alginate microspheres in PBS, and could be explained by the BSA distribution in the alginate microspheres described in the previous section. The release of IgG went through a similar trend but the initial burst release was smaller, and the overall release time was prolonged. The initial burst release of IgG was retarded to about 15 hours, and about 3-13% of IgG was released from scaffolds after 50 hours in different cases.

The protein release from scaffolds containing microspheres was affected not only by the release of proteins from the microspheres themselves, but also by the properties of the alginate hydrogels. The degradation rate, the dissolution rate, swelling effects of hydrogel scaffolds, and the diffusion rate of protein though hydrogels could influence the protein release kinetics. The degradation rate of ionically crosslinked alginate polymer is slow, rather calcium diffusion out of the hydrogels and alginate dissolution occur first *in vitro* [87, 129]. Given the two-week experimental time, the weakening of alginate cylindrical scaffolds is mainly caused by diffusion of water molecules into gels and exchanges of sodium and calcium ions, which leads to swelling of and dissolution of hydrogels. Changes in the physical properties of alginate hydrogels, such as weakening in strength and enlarged pore size, could accelerate the diffusion rate of proteins from hydrogels. However, it is obviously that the diffusion rate of proteins was effectively reduced and remained stable by embedding microspheres in alginate gels. It is possible that the alginate cylindrical scaffolds have a highly crosslinked exterior, which sufficiently lowers the permeability of gels, or the transport of proteins inside the gels was not notably affected by the ion diffusions and alginate dissolution. Since the dissolution rate of alginate and the diffusion rate of protein were not investigated (either in PBS or in TBS plus 2 mM $CaCl_2$ at 37 °C) in the current study, the contribution of each cause cannot be concluded in this study. Nevertheless, it has been reported that proteins with high molecular weight and radius, such as IgG, have a slower diffusion rate in water [131], and the alginate hydrogels crosslinked by highly concentrated $CaCl_2$ showed low membrane permeability and a more prolonged release rate [152, 176]. Therefore, it can be assumed that a higher degree of crosslinking of alginate hydrogels has

the ability to hinder the transport of proteins inside the gel but the movement of proteins is also based on the properties of proteins.

The causes of differences in the release profiles of BSA and IgG can be summarized in following points. First of all, the changes in preparation process of alginate microspheres and the nature of proteins that have been mentioned before. Secondly, the release of BSA-loaded alginate scaffolds was examined in DMEM and in TBS + 2 mM $CaCl_2$ for IgG-loaded scaffolds. Although the concentration of $CaCl_2$ is similar in these two release media, the sequestration of crosslinking calcium ions by different anions in DMEM accelerates the swelling and dissolution rate of microspheres and scaffolds. Thirdly, the BSA-scaffolds were gelled in 1M $CaCl_2$ for 12 hours, compared with 15 minutes for IgG-scaffolds. The long gelation time may induce the release of BSA during storage time, which leads to loss of BSA during processing. As discussed before, the external gelation methods involving soaking in $CaCl_2$ solution cause internal polymer rearrangements of alginate and protein; thus, the BSA molecules are more likely to be relocated close to the surface of microspheres. The internal structural changes in alginate microspheres maybe be represented in the release profiles of BSA. Lastly, the BSA-microsphere-incorporated scaffolds were prepared with dried microspheres, and the IgG ones were prepared with wet microspheres. It has been documented that dried alginate microspheres swell more substantially than wet ones in PBS [134]. The large swelling rate of dried microspheres could destroy the internal polymer network of gelled alginate scaffolds. The rupture and dissolution of BSA-alginate scaffolds could be expedited by the volume expansion of microspheres. As a result, the IgG-loaded microsphere-incorporated alginate scaffolds exhibited a lower initial burst effect and a sustained release.

Chapter 5. Summary, conclusions, and future work

5.1. Summary and conclusions of research

Alginate microspheres have been prepared using emulsification and external gelation methods. Various emulsification parameters, such as $CaCl_2$ concentration, stirring speed, total emulsion volume, and alginate-to-oil ratio, were investigated to produce small and uniform alginate microspheres. All alginate microspheres produced have a spherical shape and smooth surface; and the size and size distribution of microspheres can be controlled by using different process parameters.

Alginate microspheres produced using large emulsion volume and smaller dispersing force leads to large mean diameters and wide size distribution. The size analysis and SEM photos of microspheres suggest that high crosslinking degree and dispersing force can increase the probability of fabricating small and uniform microspheres.

Two proteins, BSA and IgG, were chosen as model proteins for preparing protein-loaded alginate microspheres. BSA was used as a reference for small proteins with low p*I* value and IgG was used as a reference for large proteins with high p*I* value. Protein-loaded alginate microspheres were successfully prepared, and two protein loading methods, incubation and incorporation, were studied using BSA. It has been established in this study that the incorporation method can achieve high encapsulation efficiencies for both BSA and IgG, and that the incubation method is not suitable for low p*I* proteins. The protein loading method should be chosen for the chemical properties of protein so that the retention of proteins by alginate polymer can be maximized.

The effects of $CaCl_2$ concentrations and protein loading methods on the release kinetics of BSA were also examined *in vitro*. The BSA release profiles of alginate microspheres loaded with incubation methods had a more substantial initial burst followed by a more rapid release, comparing to BSA loaded by incorporation methods, which further emphasizes that incubation loading methods are not suitable for BSA. Increasing the $CaCl_2$ concentration for external gelation can produce more stable microspheres, and thus, lengthen the BSA release time. On the other hand, IgG release from small and uniform alginate microspheres was slow. This sustained IgG release was possibly due to improvement in uniformity of microspheres, evenly dispersed

IgG in alginate solution prior to emulsion process, and higher degree of alginate-protein interaction.

The effect of chitosan coating on the BSA and IgG release was also studied *in vitro*. In all cases, chitosan coating reduced the initial burst and retarded the protein release. The ion contents in the release media also have influences on the *in vitro* release behaviours of proteins. It is clear that release medium containing ions with high-calcium affinity can accelerate protein release.

The release of BSA and IgG from alginate scaffolds containing incorporated microspheres was observed. Results suggest that the release of protein relies on a combined diffusion mechanism of protein diffusion from microspheres and protein movement within the scaffolds. Therefore, the dissolution, swelling, and diffusion properties of both alginate microspheres and hydrogels are critical for the release kinetics. It was observed that scaffolds prepared using highly crosslinked wet microspheres, optimal scaffold gelation time, and high crosslinking density can lower the initial burst effect and achieve prolonged protein release.

5.2. Future work

As the emulsification and external gelation process has been proven to produce spherical alginate microspheres, the optimal emulsifier concentration, the $CaCl_2$ concentration, the stirring speed, and the gelation time should be further investigated to ensure the highest productivity and encapsulation efficiency of protein-loaded alginate microspheres. The influences of chemical properties of alginate such as M/G ratios and molecular weights on the dissolution and swelling rates remain to be examined, since the protein release mechanism depends on the stability of microspheres. External gelation methods are known to change the internal alginate and protein distribution in microspheres upon gelation, so the internal structure of prepared alginate microspheres should be studied to determine the optimal gelation methods. Also, the current study demonstrates that chitosan coating of alginate microspheres can control the protein release. Therefore, the effects of chitosan with different molecular weight on the protein release kinetics need to be studied to gather more data on how to use chitosan coatings to more precisely control the protein release.

It has been shown that embedding protein-loaded microspheres within alginate scaffolds has the ability to reduce the initial burst and retard the protein release. Results reveal that protein

release from this kind of scaffold strongly relies on the properties of both the microspheres and the larger scaffolds. It can be concluded that the swelling and dissolution of alginate in microspheres and scaffolds have interactive effects on each other. This type of release behaviour imposes complications on controlling protein release; from another point of view, the protein release kinetics can be doubly controlled by optimizing the properties of both microspheres and scaffolds. As a consequence, the effects of percentages of microspheres in scaffolds, the chemical composition of scaffolds, the pore size of microspheres and scaffolds, the internal network of scaffolds, and the gelation methods on controlling the protein release should be further investigated. Also, the release medium used for *in vitro* studies should be considered carefully, and *in vitro* studies should be designed to closely mimic *in vivo* conditions. Due to the nature of proteins, different proteins represent different release kinetics. Strategies to encapsulate multi-proteins in alginate microspheres could be investigated to deliver multiple proteins at different time point and concentration in a controllable manner.

In order to be sure that this technology will ultimately have useful clinical applications, the bioactivity of the encapsulated protein should be examined. Since the protein was added into alginate solution before emulsion and alcohol washing processes in the incorporation loading method, the possibility of denaturation or breakdown of the protein exists. The biological function of a protein is strongly dependent on the native structure of the protein, and therefore, it is necessary to test the stability and biological activity of the released protein. The protein can be isolated from an *in vitro* release assay and the molecular weight of the released protein can be determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. If the breakdown of protein is not observed, an antibody assay can be used to analyze the specific biological activity of the protein. Other indirect testing method, for example, using the protein loaded microspheres in *in vitro* or *in vivo* bioassay experiments, can also be used. For example, the protein biological functions can be measured by analyzing the ability of the released protein to carry out known cellular functions or other aspects of the tissue regeneration process that are expected from the released protein.

Once the release profiles of the model protein from optimized alginate microspheres and the protein bioactivities are carefully studied, alginate microspheres or scaffolds loaded with a therapeutic protein could be implanted for *in vivo* studies. The effects of the delivered functional

protein in the target site should be examined. The activities of protein synthesis, the inflammation response, and the growth of tissues at the implantation site should be monitored to test the functions of the protein carrier.

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